CENTRO INTERNACIONAL DE ESTUDOS
DE DOUTORAMENTO E AVANZADOS
DA USC (CIEDUS)

TESIS DE DOCTORADO

DETECTION OF EMERGING ENTERIC VIRUSES IN MOLLUSC AND CLINICAL SAMPLES

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ESCUELA DE DOCTORADO INTERNACIONAL

PROGRAMA DE DOCTORADO EN
AVANCES EN BIOLOGÍA MICROBIANA Y PARASITARIA

SANTIAGO DE COMPOSTELA

2019

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DETECTION OF EMERGING ENTERIC VIRUSES IN MOLLUSC AND CLINICAL SAMPLES

D. Jesús López Romalde

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Parte del trabajo realizado en esta tesis doctoral fue financiado por el proyecto 10MMA200010PR de la Xunta de Galicia, por el Grupo de Referencia Competitivo GRC2014/007 del Plan Gallego, y por el programa IACOBUS del Grupo Estratégico Europeo de Cooperación Territorial Galicia / Norte de Portugal para una beca de investigación.

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"En el arte, nada que merezca la pena se puede hacer sin genio; en ciencia, incluso una capacidad muy modesta puede contribuir a un logro supremo"

(Bertrand Russell)

"En la investigación es incluso más importante el proceso que el logro mismo"

(Emilio Muñoz)

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A mi abuelo

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AGRADECIMIENTOS

A lo largo de esta etapa han sido muchas las personas que de una manera u otra me han ayudado. De hecho, aunque deliberadamente he dejado este apartado para el final, llevo mucho tiempo escribiéndolo hacia mis adentros, pensando en como hacer para poder devolver de alguna manera todo ese cariño que me habéis mostrado.

En primer lugar, agradecer al profesor ***Jesús L. Romalde*** la oportunidad de realizar la tesis en este grupo de investigación. Todavía recuerdo cuando entré en chándal y bastante corto de tiempo a su despacho para hacer un supuesto TAD que luego se convirtió en doctorado porque mi plan ya no incluía ese trabajo. Creo que ahí ya di la primera pista de lo despistado que soy. Gracias por la paciencia y sobre todo gracias por haberme ayudado en mi formación como científico.

En segundo lugar, agradecer a los demás profesores y personal que forman el grupo de investigación. A la profesora ***Alicia E. Toranzo***, y al profesor ***Juan L. Barja***, por ser ambos un ejemplo a seguir, la ilusión que demuestran por la investigación es envidiable y solo es comparable a la capacidad de Juan de cambiar los nombres o simplemente decir un "Flanagan" genérico que engloba prácticamente a toda la humanidad conocida. A la profesora ***Beatriz Magariños***, que, aunque no fuera su "predoc", siempre se mostró interesada en la evolución de mi trabajo, además de alegrar los cafés con sus debates que terminan con un "…O no!?" que sentencia cualquier opinión que esté a la orden del día en ese momento. A ***Celsa,*** porque creo que sin ti el departamento sería un caos y porque nadie evita de forma tan buena las preguntas sobre los días de llegada de material, eres una gran profesional que hace funcionar esta pequeña familia. A ***Berta***,

porque no hace ciencia, hace magia, de otra forma es inexplicable como puedes sacar todo lo que te pedimos en el tiempo en que lo haces. Siempre tienes una sonrisa para devolvernos y algo de tiempo para aguantar nuestros problemas, que nunca son pocos.

Por otro lado, también quiero agradecer a toda la gente del grupo de investigación que ha estado conmigo día a día. A la doctora ***Sabela Balboa***, porque no exagero nada diciendo que sin ti esta tesis no sería posible, eres una gran investigadora siempre dispuesta a echar una mano en lo profesional y una gran amiga siempre dispuesta a echar una mano en lo personal, gracias por no dejarme claudicar. A la doctora ***Ana Diéguez***, al igual que Sabela, alguna castaña me has sacado del fuego, siempre estas ahí cuando se te necesita, y porque no decirlo también para poner orden si es necesario, sacando a la luz ese carácter que solo una gran madre puede demostrar. Al doctor ***Javier Dubert***, porque eres una persona increíble, que le pone muchas ganas a todo lo que hace, no tengo ninguna duda de que te ira bien en todo lo que te propongas y tampoco tengo duda de que me lo contarás con alguna cerveza de por medio. Al doctor ***Aide Lasa***, porque es muy difícil conocer a gente como tú, porque siempre has estado ahí para intentar solucionar el mundo conmigo y porque eres un gran amigo, sin ti esta etapa no sé si sería posible, lo que sé es que no sería tan divertida. A la doctora ***Noemí Buján***, porque con tú fantasía, unicornios y alegría haces que este microcosmos funcione, y de paso que nadie se quede sin pagar ni una deuda. Al doctor (en potencia) ***Diego Gerpe***, ha sido una suerte compartir la etapa de tesis contigo, solucionando problemas y arreglando nuestras vidas entre caña y caña. Eres un gran científico "gallu" siempre podrás contar conmigo para lo que quieras. A ***Rubén Barcía***, porque has entrado pisando fuerte, y estoy seguro de que vas a dejar huella ya sea como investigador, historiador o (quien sabe) eurodiputado. A ***Beatriz Santos*** porque también has entrado fuerte, eres uno de esos relevos con los que estoy convencido que este grupo queda en buenas manos. A ***Sergio***

Rodriguez, porque estoy seguro de que puedes con todo lo venga, eres una persona brillante y fuerte como pocas. Sabes que, cuando vuelvas, aquí siempre está y estará tu casa.

A mis compañeros de poyata. Al doctor ***David Polo***, porque fuiste uno de los que me enseño "a como no estar en el laboratorio", escribiendo esta tesis no entiendo como no me llevé más gritos. Eres un gran investigador, siempre informando sobre que es lo nuevo dentro del mundo de la virología y demostrándome que aunque tenga una tesis entre manos sigo en el "A, B, C" de la ciencia. Al doctor ***Miguel A. Fernández***, porque a pesar de que nuestros inicios fueron como poco turbulentos, me has demostrado ser una gran persona, somos la prueba fehaciente de que el roce hace el cariño. Me gusta pensar que el orden que me has pegado es directamente proporcional al caos que yo te he contagiado. A la doctora ***Imen Ouardani,*** se echa de menos ese idioma inventado tan característico que nos daba para semanas de bromas. A ***Alberto Lema*** por ser de ese tipo de gente con la que se conecta rápido, de las que ponen buena cara, aunque le pidas un "estadístico" un viernes a ultima hora. Estoy seguro de que nos quedan muchas anécdotas por contar, siempre que tu agenda de meriendas lo permita.

También quiero agradecer a esas personas que me han ayudado desde fuera de las cámaras, probetas y poyatas. A mis amigos de Santiago, ***Brais*** y la doctora ***Olalla***, esa pareja irreverente que me ha puesto en mi sitio cuando lo necesitaba y me ha aguantado también cuando solo quería ver el mundo arder. A mis compañeros de equipo ***Julio (Luga)*** y ***Alex***, porque no sabéis hasta que punto me ha ayudado el desconectar de la realidad con vosotros, y por haber aguantado innumerables días de estrés con tanta paciencia. A mis amigos de toda la vida ***Roberto (Bob), Alejandro (Rodri), Daniel, Jaime, Germán, Marcos***, el otro ***Alejandro (Piki***) y ***Ricardo*** porque para bien o para mal, habéis estado ahí siempre como esa familia que se elige, que, como todas, no es ni mucho menos perfecta. Aunque de forma

especial quiero agradecer a ***Bob*** y ***Rodri***, porque pocas personas en el mundo me conocen como lo hacen ellos y porque siempre han sido una constante en mi vida, dispuestos a aguantarme sea cual sea la hora, situación y, por que no decirlo, estado. A ***María***, por demostrarme que madre hay mas que una y tratarme como uno más junto a ***Roberto***, sin el cual las comidas de verano no serían tan divertidas.

Obviamente he de incluir a mi familia, porque sin ellos no seria como soy hoy (sea lo que sea que eso signifique). A mi ***madre***, esa persona caótica que siempre está a mil cosas, aunque me deje claro que la principal es su preocupación por mi. A mi ***abuela***, porque a su forma me ha demostrado su cariño desde niño. A mis tíos ***Josecho, Mari, Luis*** y ***Flora*** porque siempre me han demostrado su cariño y sé que nunca me faltarán. A mis primos ***Quique, Luis*** y ***Jose,*** por ser ejemplos de vida cuando yo era el pequeño de la familia. Aunque de forma muy especial a Jose por actuar a la vez de hermano mayor y escuchar todas mis penas (o fingir cara de hacerlo), te dedicaría un apartado entero pero los demás se enfadarían. A mi ***abuelo***, a quien esta tesis está dedicada, porque sin su ejemplo nada en mi vida hubiese sido posible. Y por supuesto a mis otros tíos y primos ***Jose, Ana, Nico*** y ***Carolina***, porque me han demostrado su cariño y apoyo a lo largo de estos años.

Por último, aunque para nada menos importante, a la mayor experta en plata conocida y mejor persona que he podido conocer, la doctora (también en potencia) ***Ana***, por estar siempre ahí cuando te necesito y por no dejar que la situación me desbordase, gracias por ser mi compañera en esta etapa y de forma anticipada por las que están por venir.

Probablemente alguno se me ha quedado en el tintero, habéis sido tantos en esta etapa que necesitaría otra tesis para poneros, pero sabed todos que si alguno de vosotros no estuviera ahí cuando lo necesitaba esto no seria posible. De corazón ¡GRACIAS!

PUBLICATIONS

Publications included in the present doctoral thesis:

Mesquita JR, Oliveira D, Rivadulla E, Abreu-Silva J, Varela MF, Romalde JL, Nascimento MSJ (2016) Hepatitis E virus genotype 3 in mussels (*Mytilus galloprovinciallis*), Spain. Food Microbiology, 58: 13-15.
Journal Impact Factor 2016: 3.7. Q1, 9/129 in Food Science & Technology (top decile).

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Journal Impact Factor 2018: 3.811. Q2, 11/36 in Virology.

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Rivadulla E, Varela MF, Romalde JL (2019) Epidemiology of Aichi virus in fecal samples from outpatients with acute gastroenteritis in northwestern Spain. Journal of Clinical Virology (under revision).
Journal Impact Factor 2018: 3.02. Q2, 17/36 in Virology.

Other publications related with the present doctoral thesis:

Varela MF, Hooper AS, Rivadulla E, Romalde JL. (2016) Human sapovirus in mussels from ría do Burgo, A Coruña (Spain). Food and Environmental Virology, 8: 187-193.
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INDICE

SUMMARY

The present doctoral thesis brings novel information on the study of Aichi virus and Hepatitis E virus, both emerging enteric viruses causing acute gastroenteritis and hepatitis worldwide. This investigation was focused on the prevalence, quantification and genetic diversity observed in Galicia, Spain. With this purpose, collected shellfish samples from harvesting areas in the *rías* were employed, as well as clinical stool samples of people suffering acute gastroenteritis in the metropolitan area of A Coruña. Results reveal a significant presence of HEV and, at a lesser extent, AiV in Galician harvesting areas and population. All the detected HEV corresponded with genotype 3e, whereas genotypes A and B of Aichivirus were observed. Interestingly, a genotype drift was detected for Aichi virus related with the age of the patients and probably associated with changes in their feed habits. All these results reinforce the urgency of adequate viral controls for shellfish and harvesting areas not only based on bacterial indicators.

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RESUMEN

La presente tesis doctoral aporta nueva información en el estudio de Aichi vius y el virus de la Hepatitis E, ambos virus entéricos emergentes que causan gastroenteritis y hepatitis aguda en todo el mundo. Esta investigación se centró en la prevalencia, cuantificación y diversidad genética en Galicia, España. Con este propósito, se recolectaron muestras de moluscos bivalvos en zonas de cultivo de las rías gallegas, así como muestras clínicas de heces de pacientes afectados por gastroenteritis aguda en el área metropolitana de A Coruña. Los resultados obtenidos muestran una significativa presencia del virus de la Hepatitis E y, en menor medida, Aichi virus en las zonas de recolección y la población gallega. Todos los HEV detectados se correspondieron con el genotipo 3e, mientras que se observaron los genotipos A y B de Aichivirus. Es interesante destacar que se detectó una deriva en el genotipo de Aichivirus relacionada con la edad de los pacientes y probablemente asociada con cambios en sus hábitos alimenticios. Todos estos resultados refuerzan la urgencia de controles virales adecuados para moluscos bivalvos y las áreas de cultivo no solo basadas en indicadores bacterianos.

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RESUMO

A presente tese doutoral achega nova información no estudo de Aichi vius e o virus da Hepatite E, virus entéricos emerxentes ambos que causan gastroenterite e hepatite aguda en todo o mundo. Esta investigación centrouse na súa prevalencia, cuantificación e diversidade xenética en Galicia, España. Con este propósito, mostras de moluscos bivalvos foron recollidas en zonas de cultivo das rías galegas, así coma mostras clínicas de feces de pacientes afectados por gastroenterite aguda na área metropolitana da Coruña. Os resultados obtidos mostran unha significativa presenza do virus da Hepatite E e, en menor medida, de Aichi virus nas zonas de recolección e a poboación galega. Todos os HEV detectados correspondéronse có xenotipo 3e, mentras que observáronse os xenotipos A e B de Aichi virus. É interesante destacar a deriva no xenotipo de Aichi virus relacionada ca idade dos pacientes e probablemente asociada a cambios nos seus hábitos alimenticios. Todos estes resultados reforzan a urxencia de controis virais adecuados para moluscos bivalvos e as áreas de cultivo non so basadas en indicadores bacterianos.

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ABBREVIATIONS AND ACRONYMS

°C	Celsius degree
AiV	aichivirus
CDC	Center for Disease Control
CV	coxsakievirus
DNA	deoxyribonucleic acid
ds	double stranded
dPCR	digital polymerase chain reaction
ECDC	European Centre for Disease Prevention and Control
ELISA	enzyme-linked immunoSorbent assay
EM	electron microscopy
etc	etcetera
EU	European Union
EV	echovirus
EWS	early warning system
FBD	foodborne diseases
FBO	food-borne outbreaks
HAsV	Human astrovirus
HAV	hepatitis A virus
HEV	hepatitis E virus
HSC	heat shock cognate protein
HSPG	Heparan sulfate proteoglycan
ICTV	International Committee on the Taxonomy of Viruses
IEM	immune electron microscopy
Kb	kilobase
kGy	kiloGray
min	minute
MPa	megaPascal
MPN	most probable number
nm	nanometre
NoV	norovirus

NS	nonstructural protein
NTP	nucleoside triphosphate
ORF	open reading frame
PCR	polymerase chain reaction
P	protruding domain
PV	poliovirus
qPCR	real-time/quantitative polymerase chain reaction
RdRp	RNA-dependent RNA polymerase
RER	rough endoplasmic reticulum
RTE	ready to eat food
RT	reverse transcription
RNA	ribonucleic acid
RT	reverse transcription
RV	rotavirus
S	shell domain
s	second
SaV	sapovirus
ss	single stranded
UK	United Kingdom
USA	United States of America
UV	ultraviolet
VP	viral capsid protein
VPg	viral protein genome-linked
WHO	World Health Organization
WWTP	wastewater treatment plant

1. INTRODUCTION

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1. INTRODUCTION

1.1 Food-borne diseases. A worldwide issue

Ingestion of contaminated food results in food-borne diseases (FBD) that could be caused by biological pathogens (bacteria, viruses, prions, protozoa and other parasites) or chemical and preformed toxins (Epp and Parker, 2009; Kalyoussef and Feja, 2014). Although nowadays the routes of transmission in the food chain are well known, this kind of knowledge is relatively recent.

At the beginning of 19th century, some infectious diseases as syphilis or smallpox were accepted to be contagious, but there was an extended debate around the origin of certain illnesses such as cholera. The predominant model to explain its transmission, was the "miasma" theory. According to that theory, miasmas were a set of fetid emanations from impure soils and waters released into the air that were toxic for the people (Halliday, 2001; Julia and Valleron, 2011; Tulodziecki, 2011).

The theory was accepted throughout the century. However, the breaking point came during the London cholera outbreak in 1853-1854 when John Snow located the homes of affected individuals and the public water sources, and concluded that the higher number of deaths occurred near a public pump on Broad Street (Koch and Denicke, 2009; Newsom, 2006; Ramsay, 2006). This correlation between cholera incidence and the public pump, confirmed his theory

about the cholera transmission by contaminated water, instead the popular belief that the illness was air-borne (Shiode et al., 2015). Finally, the theory of water transmission was accepted in 1866 (Ramsay, 2006). Since then, methods for food-borne pathogens detection have improved and the transmission routes are clearer.

Within the FBD agents, biological pathogens are those that concentrate more efforts and public concern (Achenson, 1999; Carter, 2005). Around the world the main biological pathogens detected are Rotavirus (RV), Norovirus (NoV), Hepatitis A virus (HAV) *Campylobacter spp*. and *Salmonella* (Hoffman et al., 2012).

However, the overall incidence of FBD is difficult to determine, since there is a number of situations that must occur to report an FBD: first of all, the affected individual or individuals must need medical care. Then, health care professionals must request a clinical sample, with the agreement of the patient. After that, the investigators must identify the causative agent and finally, the diagnostic results must be reported and further analyzed (Figure 1).

If any of these steps is missed, the FBD will be undiagnosed or unreported and the epidemiological data will be lost (Hoffman and Scallan, 2017). Furthermore, there are foods made with multiple ingredients, which connected with the bad memory of consumers about the foods eaten, and the long incubate periods of some diseases, make the identification of FBD pathogens more challenging.

Annual estimations establish that food-borne pathogens affect one-third of the world population and cause approximately 48 million

illnesses resulting in 127,839 hospitalizations and 303 deaths (Hoffman and Scallan, 2017). In the European Union (EU) and surrounding countries about 23 million people become ill because FBD, even more, this number has been increasing since the first stages of 21st century (WHO, 2015; EFSA/ECDC, 2016). The largest proportion of this FBD burden (about 40%) is among children under 5 years old (Dhama et al., 2013; Kalyoussef and Feja, 2014; Schlundt et al., 2004).

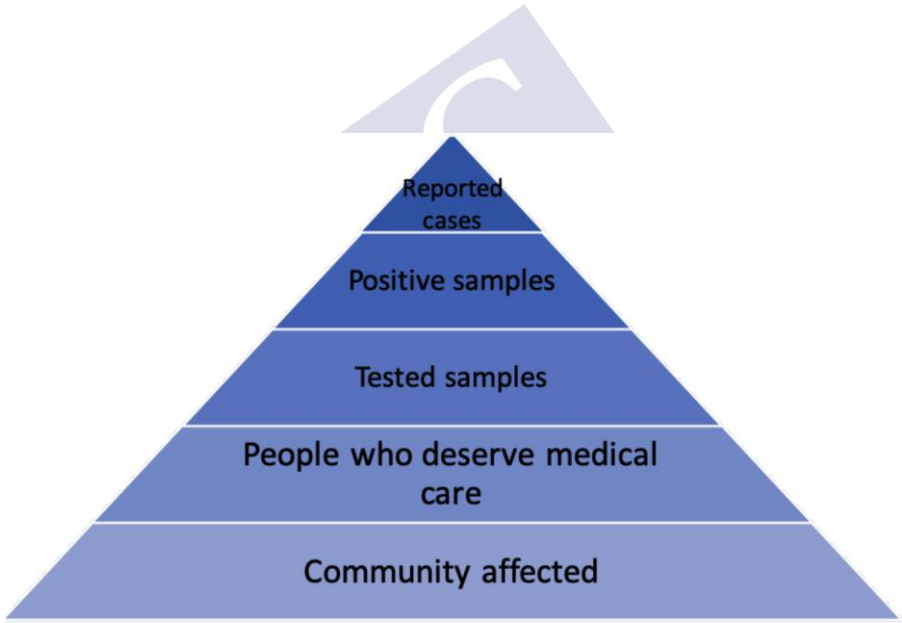


Figure 1. FBD surveillance pyramid (Adapted from Hoffman and Scallan, 2017).

The FBD symptoms vary and could persist from 8 h to several days or weeks, depending on the initial contamination dose and on the pathogen characteristics. The gastrointestinal tract is usually the most

affected organ and the most frequent symptoms diagnosed include nausea, vomits, and diarrhea. Nonetheless, some pathogens can cause extraintestinal alterations, especially on immunocompromised individuals. *Listeria monocytogenes*, for example, causes septicemia, stillbirths, and other extraintestinal problems in pregnant woman, elderly persons, or immunosuppressed people (Rees et al., 2017). Furthermore, there are organisms that usually cause gastrointestinal diseases but occasionally cause extraintestinal illnesses, such as *Yersinia enterocolitica* that causes diarrhea and abdominal pain, but also could produce arthritis (Fredriksson-Ahomaa, 2017). In addition, other enteric pathogens cause extraintestinal infections routinely, like HAV inducing its symptoms in the liver (Cuthbert, 2001).

The economic implications of FBD are also important. For instance, only in Netherlands reported cost for NoV and HAV were about 106 € millions and 900,000 €, respectively, each year (Mangen et al., 2015). It is estimated that FBD cause economic losses ranging from $ 55.5 to $ 93.2 billion per year in United States (USA) (Scharf, 2015).

Nowadays, globalization has promoted new improvements on the transport logistic and conditions but also have opened new transmission routes among human populations, enabling the pathogens to survive longer and reach the consumers in an infective form. Food contamination could occur in several phases, including the places where animals and plants are grown/cultured and harvested, during the transportation and product processing, or as a result of cross-contamination from food-handlers or consumers (Hall et al., 2014).

1.2 Enteric viruses

1.2.1 Historical approach

For a long time through history, the transmission of diseases was thought to be caused by witchcraft, earthquakes, religious forces, comets and meteors (Charkrabarti, 2010; Karamanou et al., 2012). Only recently, and due the technological advances, we were able to characterize the etiological agents and to study their epidemiology.

Despite that viruses are the top causes of FBD globally (Havelaar et al., 2015), the fact that some of these pathogens could not be isolated *in vitro* hindered their identification. Consequently, epidemiological efforts and data have been mostly associated with pathogenic bacteria. The first documented viral outbreak associated with food was in 1914. Transmitted through raw milk, the causative agent was recognized as Poliovirus (PV) (Jubb, 1915). After that, more milk- and water-borne outbreaks were reported (Cliver 1969; Mosley, 1967) and PV was isolated and cultivated *in vitro* in 1949 (Enders et al., 1949), allowing the development of a vaccine (Sattar and Tetro, 2018). Since then, a sustained and widespread immunization against this pathogen reduced drastically the poliomyelitis outbreaks. Even more, nowadays PV spread by food or any other routes has virtually disappeared (Sattar and Tetro et al., 2018).

However, the crucial step in the study of virus as FBD agents occurred during a hepatitis outbreak that had place in the middle of

20th century. At that time, 29,000 people were affected by hepatitis E virus (HEV) in Nueva Delhi and HAV outbreaks transmitted by shellfish were reported from Sweden (Roos, 1956) and USA (Mason and McLean, 1962). These situations pointed out the necessity to study the viral sources and the importance of these "new" non-bacterial pathogens among human population.

On the other hand, in 1929 the pediatrician John Zahorsky described the "Hyperemesis heimis" or winter vomiting disease (Zahorsky, 1929). The illness was accompanied with diarrhea and affected the population every year, but the pathogen could not be identified. In March of 1963 this illness occurred in a Britain school, where 142 students suffered a gastrointestinal disease. A clinical sample of an affected student was stored at –70ºC and used later in an experiment in which some volunteers where experimentally infected. The volunteers drank a glass of sodium bicarbonate and immediately a glass of orange juice contaminated with the student sample. Although in that survey the investigators could not identify the causal agent either, they could verify the capability of this non-bacterial agent for transmission, their heat resistance and its diameter smaller than 50 nm (Clarke et al., 1972).

Five years later, in 1968, another case was reported from other school. In this case the "winter vomiting outbreak" took place in Norwalk, Ohio (USA). A similar procedure was carried out and finally in 1972, due the scientific advantages of the time, an image of the pathogen was obtained by immuno-electron microscopy (IEM) from the stools of volunteers infected with leaked feces of affected people

from the 1968 outbreak (Kapikian et al., 1972). The pathogen was described as Norwalk-virus and become the first gastrointestinal virus described (Kapikian et al., 1972; Robilotti et al., 2015).

One year later, infective particles/virions were found in duodenal mucosa of hospitalized children with acute gastroenteritis. IEM was again helpful for the characterization of the virus named reovirus-like (Bishop et al., 1973). Since 2002 until today Norwalk-virus is known as Norovirus that belongs to *Calciviridae* family and the reovirus-like is known as Rotavirus and belongs to *Reoviridae* family. Both viruses are the major causes of acute gastroenteritis worldwide.

In the last years, molecular methods for detection of difficult or uncultivable viruses became available and the number of documented food-borne virus outbreaks has increased (Koopmans et al., 2002). The new technologies and procedures like the polymerase chain reaction (PCR), the real time PCR (qPCR) and digital PCR (dPCR), which will be deeply discussed later, have improve our understanding about viruses.

The current impact of FBD on the human population and health systems is unknown and the existing data are limited to a few industrialized countries (Morris, 2011). Many of these countries, despite having some level of notification of FBD, do not include diseases of viral etiology and/or do not usually focus on food transmission as a source of infection. Furthermore, the development of a global trade without appropriate virological safety procedures, both

in industrialized and developing countries, together with the ineffectiveness of existing microbiological standards as indicators of viral contamination, illustrate the current vulnerability of the global food market. As a result, national statistics on FBD of viral etiology are difficult to obtain or present a significant underestimation of the cases (O'Brien, 2008).

1.2.2 General characteristics and routes of transmission

Viruses are obligate intracellular parasites that require an adequate host cell for propagation. The virion consists in a nucleic acid of a single stranded (ss) or double stranded (ds) DNA or RNA, and an outer protein coat called capsid. Sometimes these particles are enveloped by a lipid bilayer derived from host cell membranes and viral proteins. Depending on the presence of this structure the virions are classified as enveloped or non-enveloped. In addition, the distribution of viruses in families and orders depends on the virion shape, size, genome composition and replication mode (Bosch et al., 2018; King et al., 2012).

Because virions are inert particles, unlike bacteria they can not replicate in food or water during the processing, transporting and storage. The capability of food to serve as a vehicle for enteric viruses depends on virus stability, degree of initial contamination (even one infective particle has a significant probability for causing disease), the method used for food processing and storage, viral dose needed, and the susceptibility of the host to pathogen (Koopmans et al., 2002). The

viruses need to be extracted from food (or water), prior their amplification by molecular methods, in order to reduce possibe inhibitors present in these matrices that could limit the sensitivity of viral assays (Gerba, 2006).

Virions are stable to different environmental conditions including pH, low temperatures, and some enzymes, especially those that are found in the human gastrointestinal tract (Jaykus, 2000). Because FBD viruses are transmitted via the fecal-oral route (Figure 2), they spread easily in places where individuals are in close proximity or are sharing the same water pumps or food. Consequently, most acute gastroenteritis outbreaks were documented in schools, recreational camps, hotels, hospitals, orphanages, nursing homes and restaurants.

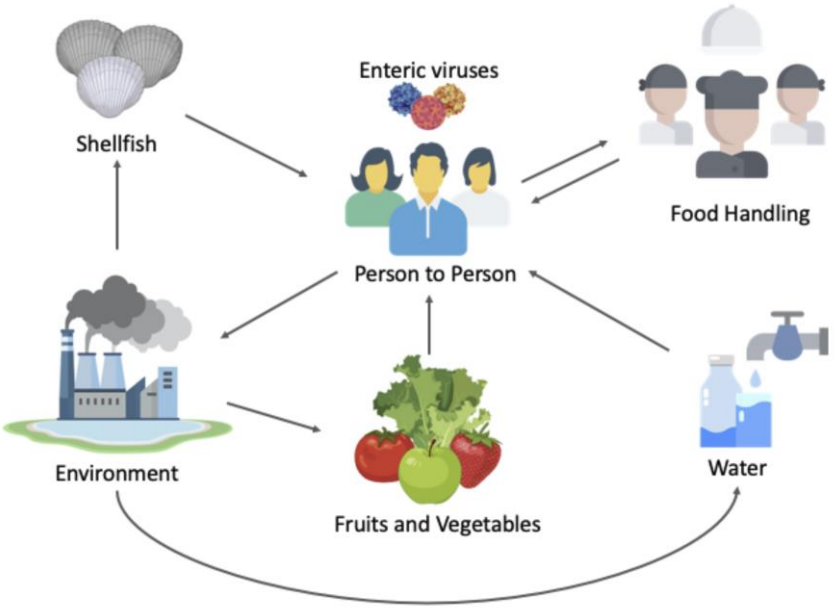


Figure 2. Scheme of enteric viruses transmission through the fecal-oral route.

Over 45 % of food-borne outbreaks (FBO) reported to CDC between 1998 and 2008 were caused by food-borne viruses (Gould et al. 2013). Shellfish and water are viral vehicles that will be discussed later. However, the importance of fresh food and ready to eat food (RTE) is being increasingly recognized.

A number of FBO associated with the consumption of contaminated fruits and vegetables occurred several times over the past years. As an example, between 1988 and 1997, the CDC reported 130 FBO linked with that kind of food showing a clear increasing tendency the following years. Most of the times, contamination of vegetables or fruits occurs before the product reaches the establishments (Koopmans et al., 2002). Depending on the type of viruses, growing season, soil composition, temperature, rainfall or resident microbiota, virions can survive in the environment for long term periods (Yates et al., 1985; Seymour and Appleton, 2001). Crops are usually irrigated with river water as well as reclaimed water, both of which could contain fecal material and, additionally, these foods could be handled by field workers or food handlers with poor hygiene practices.

In Europe, 83 outbreaks were reported between 1992 and 1999 in England and Wales, associated with the consumption of vegetables and fruit (O´Brien et al., 2000). In 2012, a FBO affected approximately 11,000 people in Germany. Epidemiological investigations found that strawberries imported from China, that were

(supposedly) cultivated with untreated wastewater, were the source of NoV contamination (Mäde et al., 2013). More recently, in 2013, frozen berries from different origin (Canada, Bulgaria, Serbia and Poland) were also linked to a HAV outbreak in Northern Italy (Rizzo et al., 2013).

On the other hand, it is also necessary to highlight the role that RTE food plays in FBO. Epidemiological data showed that the transmission of food-borne viruses through food handlers and consumers is extended in RTE foods. Indeed, the poor personal hygiene of infected food handlers is one of the most cited risk factors in FBO (Greening et al., 2016). RTE foods are defined as eatable products without need of washing or cooking by the consumer or the food service establishment prior consumption (Public Health Service, 1999). Consequently, contamination of RTE foods can only occur during the post-processing step.

Food handlers and consumers can spread enteric viruses to foods from other foods, contaminated surfaces or from other food handlers that could be symptomatic or asymptomatic. However, human fecal material is the usual source of contamination (Richards, 2001). As an example, between may of 1998 and June of 1999, three gastroenteritis outbreaks caused by NoV occurred in Melbourne, Australia. It was in a restaurant where food was served on platters and eaten with hands from a shared dish. In the three outbreaks the virus appeared to be introduced by consumers or food handlers, opposed to a long-term food reservoir. Two outbreaks were linked to guests, and

the third was suggested to be originated by a food handler that was positive for NoV (Marshall et al., 2001).

Another study carried out in Denmark, reported that between 2006 and 2011, of 191 FBO caused by members of the *Caliciviridae* family (189 Nov and 2 Sapovirus [SaV]), 51 were due to contaminations during food production. Nevertheless, 55 FBO were originated after the production, and linked to consumers who contaminated the food in self-service buffets. The same study showed that food handlers were responsible for 64 FBO, 41 of which were originated by asymptomatic individuals who were in incubation/recovery period, or had contact with sick people (Frank et al., 2015).

Nonenveloped viruses such NoV, SaV or HAV, survive better than enveloped ones on skin. Consequently, contaminated hands frequently play an important role in virus spread, acting as virus donors and recipients. The importance of a good hand hygiene previously to processing or eaten the foods, needs to be emphasized (Sattar and Tetro, 2018).

1.2.3. Main enteric viruses

A large amount of viruses have been identified as FBD etiological agents, such as RV, NoV or human astrovirus (HAstV). However other enteric viruses as SaV, or Aichi virus (AiV) are implicated in sporadic cases of gastroenteritis as well as in gastroenteritis outbreaks. On the other hand, HAV is the most

important FBO agent that causes hepatitis all around the world, but in recent times HEV has also emerged as an important hepatitis pathogen (Rein et al., 2012).

1.2.3.1. Rotavirus

Discovered in duodenal biopsies from nine children suffering acute gastroenteritis in 1973 (Bishop et al., 1973), RV is a well-known etiological agent of FBO and waterborne outbreaks worldwide, especially in child under 5 years. Its name is due the particular wheel-like appearance (Figure 3) ("*Rota*" means wheel in Latin) when observed under electron microscopy (EM) (Flewett et al., 1974).

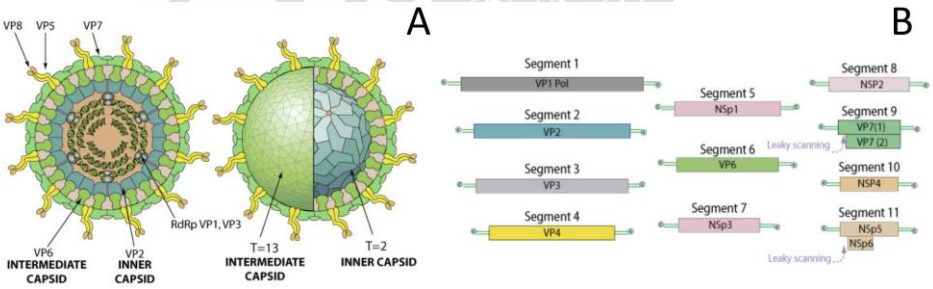


Figure 3. A) Schematic representation of RV virions particles. Virions are non-enveloped and about 70-75 nm in diameter with T=13 icosahedral symmetry in the inner capsid and T=2 icosahedral symmetry. The capsid is composed by six structural proteins (VP1-4, VP6 and VP7). B) Segmented linear dsRNA genome of RV. Contains 11 segments for 12 proteins (VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4, and NSP5/6, respectively. (Extracted from ExPASy: SIB bioinformatics resource portal)

It is a nonenveloped virus with a diameter of 70-75 nm, that belongs to the family *Reoviridae* in the subfamily *Sedoreovirinae*, and in the genus *Rotavirus* (King et al., 2012). The genome is about 18.5 Kb in size, composed by a linear dsRNA which contains eleven segments that encodes six structural proteins (VP1-4, VP6 and VP7) and six nonstructural (NSP1-NSP6) (Mattion et al., 1991).

On the basis of antigenicity and genome sequence properties, RV is classified into eight species (RVA-RVH) and two proposed ptential new species (RVI and RVJ), recently identified in Hungarian dogs and Serbian bats. Among the eight recognized species, RVA-RVC are known to infect humans, being RVA the most extended common enteric agent around the world. A genotyping system involving all 11 genome segments has been developed, assigning genotypes (G, P, I, R, C, M, A, N, T, E, and H genotypes) for each gene segment (VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4, and NSP5/6, respectively), on the basis of calculated nucleotide sequence identity cutoff values. To date, 35 G (VP7), 50 P (VP4), 24 I (VP6), 14 R (VP1), 18 C (VP2), 17 M (VP3), 28 A (NSP1), 17 N (NSP2), 19 T (NSP3), 24 E (NSP4), and 19 H (NSP5) genotypes have been described (Matthijnssens et al., 2008; Shetty et al 2014). The G1, G2, G3, G4, G9, G12 genotypes together with P[4], P[6], P[8]; and genotype combinations G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], G12P[8] are responsible for almost 90% RV infections globally (Matthijnssens et al., 2012; Li et al., 2016).

Although RV rarely produces symptomatical infections in adults, is responsible for 20-30% of all acute gastroenteritis cases that requires hospitalization in children (Lekana-Douki et al., 2015). It is also estimated that RV causes 450,000 deaths (more than 90% occurring in developing countries), more than 2 million hospitalizations and 25 million outpatients each year (Tate et al., 2012; WHO, 2007). Only in Europe RV causes 231 deaths, more than 87,000 hospitalizations, and almost 700,000 outpatients every year (Arístegui et al., 2016).

In Europe or USA, RV infections occur mostly in winter months. Meanwhile, in African countries the RV infections are predominant in dry seasons, but this is mostly because only a small proportion of population has access to safe drinking water rather than a seasonal effect (Chaudhuri et al., 1990; Kim et al., 2005). Rotavirus disease symptoms range from asymptomatic or mild watery diarrhea to acute gastroenteritis and even death due to dehydration. Incubation period is 1 to 3 days and the disease symptoms are fever, urination decrease, dry mouth and throat, respiratory illness, dizziness when standing up, abdominal pain, and vomiting for 2 to 3 days, followed by rapid dehydration that leads in electrolyte imbalance due to nonbloody diarrhea for 3 to 8 days. The sick individuals shed virus via feces and are the major reservoirs for infecting others (Alkali et al., 2012).

Since 2006, two RV vaccines exist: a pentavalent bovine–human reassortant vaccine (RotaTeq, Merck, West Point, PA, USA) and a monovalent human vaccine (Rotarix, GlaxoSmithKline

Biologicals, Rixensart, Belgium), both with similar efficacy. The routine vaccination of infants has been implemented in 95 countries globally. A reduction of RV disease after vaccination was observed but a long-term monitoring is required to confirm such results. In addition, more investigations will be needed in order to develop cost-effective vaccines with good protection levels and safety profiles (Sadiq et al., 2018).

1.2.3.2. Astrovirus

Discovered in 1975 in stool samples from children that suffered diarrhea (Appleton et al., 1975), HAstV are nonenveloped viruses of approximately 25-30 nm and a positive sense ssRNA of about 6.2-7.7 kb that belong to the *Astroviridae* family (Figure 4). The genome is divided in three ORFs: ORF1a, ORF1b, and ORF2. ORF1a and ORF1b encode nonstructural proteins while the ORF2 encodes capsid proteins. Since 2008 HAstV are divided in Classical HAstV (serotypes 1-8) and Novel HAstV (namely MLB and VA/HMO) (Vu et al., 2017). Classical HAstV-1 are the major cause of viral diarrhea after NoV and RV worldwide, and affect mainly children, the elderly and immunocompromised people (Moser and Schultz, 2005; Borrows et al., 2014). Classical HAstV-1 are the most common among population, with a prevalence of up to 90% in children by the age of 5 years (Bosch et al., 2014).

Symptomatology of HAstV consists in mild gastroenteritis, being a self-limiting disease that includes two or three days with

watery diarrhea. Vomiting is less common in HAstV than in RV or NoV. However, HAstV has been associated with encephalitis and meningitis in immunocompromised patients (Vu et al., 2016), and a relation between this pathogen and necrotizing enterocolitis infection has also observed in neonates (Bagci et al., 2010; Chappe et al., 2012).

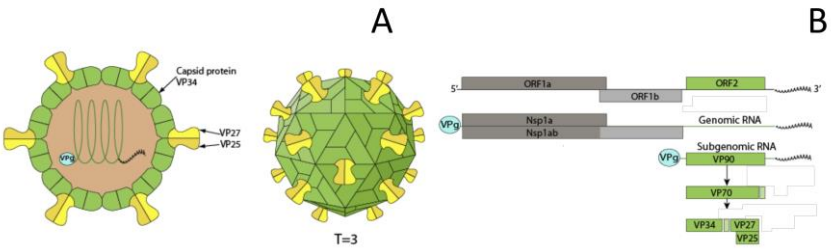


Figure 4. A) Schematic representation of HAstV virions particles. Virions are non-enveloped, spherical and about 25-30 nm in diameter with T=3 icosahedral symmetry. The capsid is assembled by N-terminal domain of VP34 and the surface spikes are attributed to dimers of the VP25/VP27 variable domain. B) Monopartite linear ssRNA (+) genome of HAstV. The genome is divided in three ORFs: ORF1a, ORF1b, and ORF2. ORF1a and ORF1b encodes nonstructural proteins while the ORF2 encodes capsid proteins (Extracted from ExPASy: SIB bioinformatics resource portal).

Regarding to seasonality, classical HAstV infections occur mostly during winter season, meanwhile novel HAstV diarrhea occurs along the whole year (Bosch et al., 2014; Cordey et al., 2017). Although some genotypes seem to be established in the same area for a long time, other lineages have been shown to emerge or re-emerge

over the time (De Grazia et al., 2016), indicating a recirculation and evolution of this pathogen among human population.

Immunity against HAstV remains unclear and some FBO were reported in expected immune populations (Koopmans et al., 2017). Furthermore, the importance of HAstV correlation with other enteric virus in coinfection has been reported (Reither et al., 2007), fact that could mask the real health importance and makes more challenging to measure its clinical burden.

1.2.3.3. Norovirus

NoV is a nonenveloped virus with a diameter of 27-40 nm. The genome is about 7.5-8.3 Kb in size, composed by a positive ssRNA that encodes three open reading frames (ORF) (Figure 5). The ORF1 encodes a polyprotein that is post-translationally cleaved into seven nonstructural proteins (NS1 to NS7), and the other two ORFs have a role on virion formation (Thorne and Godfellow, 2014). The ORF2 encodes the major capsid protein, VP1 and presents two domains, shell (S) and protruding (P), that forms the virion with the minor capsid protein VP2, encoded by the ORF3. Based on VP1 and the RNA dependent RNA polymerase (RdRp), NoV is classified in seven genogroups (GI to GVII) of which Genogroups GI, GII and GIV infect humans. Human NoV are further divided into a total of 28 genotypes (Eden et al., 2014; Lizasoain et al., 2015; Parra et al., 2017; Vinjé, 2015), but the most common is genotype GII.4 responsible for almost 80% of the FBO produced by this pathogen (van Beek et al.,

2016). However, a new genotype GII.17 has emerged worldwide (de Graaf et al., 2015), indicating a continuous recombination due to recirculation among human population.

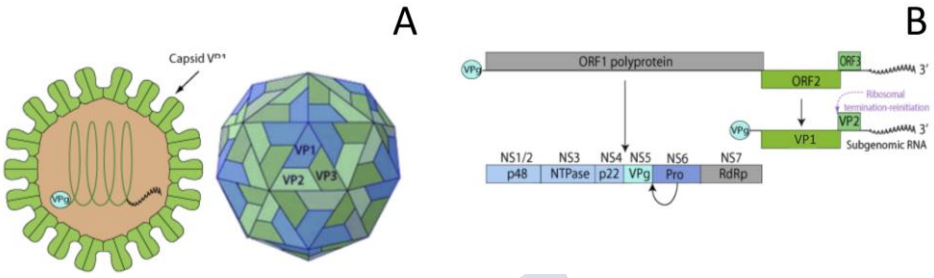


Figure 4. A) Schematic representation of NoV virions particles. Virions are non-enveloped and about 38-40 nm in diameter with T=3 icosahedral symmetry. The capsid is composed VP1, VP2 and VP3 proteins. B) Monopartite linear ssRNA (+) genome of NoV. The ORF1 encodes a polyprotein that is post-translationally cleaved into seven nonstructural proteins (NS1 to NS7). The ORF2 encodes a major capsid protein VP1 that forms the virion with the minor capsid protein VP2, encoded in the ORF3 (Extracted from ExPASy: SIB bioinformatics resource portal).

NoV causes acute gastroenteritis all around the world in people of all ages. Each year, only in US, NoV causes cause on average 19-21 million illnesses, 56,000-71,000 hospital admissions, and 570-800 deaths (Hall et al., 2013). Symptoms are similar to RV, and include nausea, vomiting, diarrhea, fever, chills, abdominal pain, and also myalgias and headache (Kaplan et al., 1982). Children and people aged over 65 are the individuals who account the majority of fatalities associated with NoV, together with immunocompromised individuals

who can become chronically infected (Hall et al., 2013; Harris et al., 2008). The incubation period is for about 24-48h and the symptoms usually last 12-72h (Kaplan et al., 1982). Nonetheless complete viral elimination could occur during 3 weeks after the resolution of symptoms, and even longer times have been reported for children under 1 year of age (Kirkwood and Streitberg, 2008; Murata et al., 2007, Rockx et al., 2002).

Although NoV causes sporadic FBO through the year, a seasonality pattern has been reported. NoV FBO tend to increase during the cooler months of the year in developed countries. In the tropical areas, something similar happens but associated to the rainy seasons (Bucardo et al., 2008; Hall et al., 2014). Infected individuals develop an immune response after infection that offers partial protection against subsequent infections, although it is of short duration (between 6 and 14 weeks) (Johnson et al., 1990; Parrino et al., 1977; Rockx et al., 2002). Nonetheless, cyclical peaks of NoV happen every few years with the emergence of new genotypes against which population immunity is inadequate (Cannon et al., 2009; Zheng et al., 2010). In the last years and under specific lab conditions, NoV could be replicated in stem cell–derived human enteroids (Ettayebi et al., 2016) and vaccines against NoV are being developed (Pringle et al. 2015; Kocher and Yuan 2015). However, there are several challenges, such as the incomplete understanding of the immunity against this virus or the fact that NoV cultivation is a time consuming and expensive methodology.

1.2.3.4 Sapovirus

Discovered in 1979 from fecal samples of children after an outbreak of gastroenteritis in an orphanage, in Sapporo (Japan), SaV belongs to the family *Calciviridae* and the virions are nonenveloped particles of approximately 30-38 nm, showing a "Star of David" morphology under EM (Chiba et al., 1979). SaV present a genome of positive sense ssRNA, with about 7.3-7.5 kb in size, comprising 2 or 3 ORFs (Figure 6). The ORF1 encodes a polyprotein containing the non-structural proteins: NS1-7 and the major capsid protein VP1. The ORF2 encodes the minor capsid protein, VP2. The ORF3 is only present in some strains (Schuffenecker et al., 2001; Farkas et al., 2004).

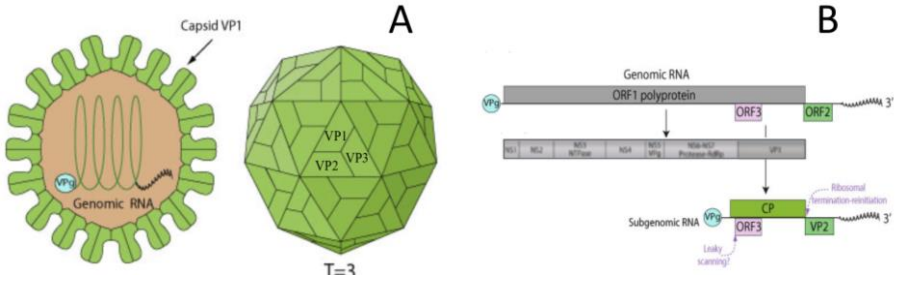


Figure 6. A) Schematic representation of SaV virions. Virions are non-enveloped and about 30-38 nm in diameter with T=3 icosahedral symmetry. The capsid is VP1, VP2 and VP3 proteins B) Monopartite linear ssRNA(+) genome of SaV. The ORF1 encodes a polyprotein containing the non-structural proteins: NS1, NS2, NS3, NS4, NS5, NS6 and NS7 as well as the major capsid protein VP1. The ORF2 encodes the minor capsid protein, VP2. The ORF3 is only present in some strains. (Extracted from ExPASy: SIB bioinformatics resource portal).

Based on the VP1 region, five SaV genogroups (GI to GV) were proposed. The SaV genogroups GI, GII, GIV and GV are the human enteric pathogens with a total of 16 genotypes (GI.1–7, GII.1–7, GIV.1 and GV.1) (Clarke et al., 2012; Liu et al., 2016) Nevertheless, recently phylogenetic analysis using complete capsid sequences of human and animal strains have revealed 19 different SaV genogroups, adding fourteen (from GVI to GXIX) to the current classification (Scheuer et al., 2013; Oka et al., 2016; Yinda et al., 2017). SaV GI and GII are the most common genogroups observed worldwide in recent years. Among them, GI.1, GI.2 and GII.1 are the main detected genotypes (Gallimore et al., 2006; Svraka et al., 2010; Díez-Valcarce et al., 2018).

As in the case of HAstV, SaV gastroenteritis presents milder severity than RV and NoV. It is usually a self-limiting gastroenteritis disease and symptoms normally disappear after 12-72 hours (Rockx et al., 2002; Kobayashi et al., 2012). The major groups affected by SaV are also infants, children and immunocompromised individuals susceptible to more serious clinical complications (Nakata et al., 1988; Sakai et al., 2001; Wu et al., 2008). Attending to seasonality, as usually in enteric viruses, SaV incidence is higher during the cold and rainy seasons (Phan et al., 2004; Johnsen et al., 2009; Dey et al., 2012), but sporadic cases have also been reported during the rest of the year (Pang et al., 2000).

Seroprevalence rates increase with age. Affected individuals are thought to develop a short duration immunity response after SaV contamination that offers a partial protection against posterior

infections (Rockx et al., 2002). However, continuous infections with different SaV genotypes were reported, showing a continuous circulation worldwide (Harada et al., 2012).

1.2.3.5 Hepatitis A

Since the time of Hipocrates HAV infections were described, but it is in 1973 when viral particles were visualized by IEM (Feinstone et al., 1973). Six years later in 1979 *in vitro* multiplication of HAV was achieved in cells derived from kidney of *Rhesus* monkey (Provost and Hilleman, 1979). HAV belongs to the *Picornaviridae* family within the *Hepatovirus* genus. The virions are nonenveloped particles of approximately 27-32 nm. HAV has a positive sense ssRNA genome of approximately 7,5 kb (Figure 7) (Cohen et al., 1987). This genome present only one ORF encoding a polyprotein that contains 3 major regions, P1, P2 and P3, required for virion maturation. The P1 region is processed into the structural proteins VP1, VP2, VP3, and also the VP4, essential for virion formation but not present in the mature particles (Probst et al., 1999). Viral protease 3C cleaved structural proteins and is encoded in the P3 region. The P2 and P3 regions contain nonstructural proteins that are required for RNA synthesis and virion assembly. The P3 segment contains the 3A, 3B, 3C and 3D proteins. Among these, 3C is the protease responsible for most cleavages within the polyprotein (Robertson et al., 1992; Hollinger and Emerson, 2007; Costa-Mattioli et al., 2003).

Based on the VP1 region, 3 genotypes (I to III) that affect humans have been described. These genotypes have been divided in 6 subgenotypes IA, IB, IIA, IIB, IIIA and IIIB. Genotype I is the most prevalent in the world, being the subgenotype IA more common than IB. However, both subgenotypes are frequently detected in North America, South America, Europe, China and Japan (Robertson et al., 1992, Costa-Mattioli et al., 2001a, 2001b).

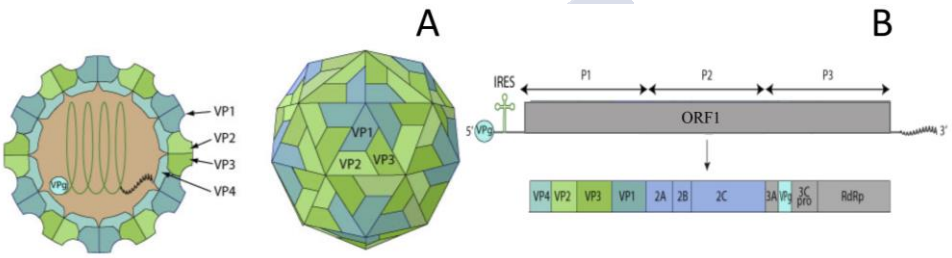


Figure 7. A) Schematic representation of HAV virions. Virions are non-enveloped particles of approximately 27-32 nm in diameter with T=pseudo3 icosahedral symmetry. The capsid is VP1, VP2 and VP3 proteins B) Monopartite linear ssRNA (+) genome of HAV. The ORF1 encodes a polyprotein that contains 3 major regions, P1, P2 and P3. The P1 region is processed into the structural proteins VP1, VP2, VP3, and also the VP4. Viral protease 3C is encoded in the P3 region. The P2 and P3 regions contain nonstructural proteins. Segment P3 contains the 3A, 3B, 3C and 3D proteins. (Extracted from ExPASy: SIB bioinformatics resource portal).

HAV is less frequent in developed regions than in developing countries where is highly endemic. Epidemiology of HAV varies from

asymptomatic infection to severe hepatitis. Clinical HAV manifestations depend on the age of the patient. In children under 6 years of age, approximately 70% of infections are asymptomatic. On the contrary, infection is usually symptomatic in 70% of adults, causing jaundice and the presence of a high number of aminotransferases in serum (Cuthbert, 2001).

The incubation period is about is approximately 30 days, with a range of 15-50 days and symptoms includes: fever, nausea, vomiting, abdominal pain, dark urine and jaundice. Myalgia, pruritus, diarrhea, arthralgia, and skin rashes can occur less frequently (Koff et al., 1992).

Regarding to seasonality, there is no definite and consistent seasonal pattern observed for HAV, although evidences point towards spring and summer peak (Fares, 2015). The asymptomatic infections among children<5 years generate long-lasting immunity and vaccination in countries where is highly endemic is not recommended (Jacobsen and Wiersma, 2010). However according to WHO guidelines, largescale HAV vaccination programs in low endemic regions are likely to be cost-effective and are encouraged to prevent population from being infected and develop disease (WHO, 2013).

1.2.3.6 Other Viruses

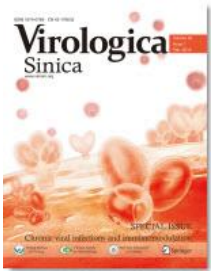
Enteric viruses such Echovirus (EV), Coxsakievirus (CV) or Coronavirus (CoV) are less common than the ones cited above but are also important gastroenteritis agents.

In 1976, an FBO due to EV was described in Pennsylvania. In that FBO, 80 cases of meningitis were recorded among people who ate coleslaw in a picnic (CDC, 1979). Twelve years later, in New York, an outbreak affected 161 individuals with unspecified illness. EV was reported as the causative agent but the source of contamination was undetermined (Altekruse et al., 1998). Regarding to CV, a FBO occurred in a day care center of the Soviet Union. The pathogen was isolated from certain items of food, surfaces of food preparing areas and some affected individuals (Sattar and Tetro et al., 2018).

On the other hand, the role of CoV as gastroenteritis agent is unknown. The virus causes acute infections of respiratory tract, although a FBO in Scotland was reported and attributed to CoV (Anonymous, 1991). Recently, CoV was also detected in stools from humans and animals with diarrhea, but more studies are needed to know is real implication as FBO agent (Corman et al., 2018).

1.3 Aichi virus.

Article 1. Rivadulla E, Romalde JL (2019) A comprehensive review on human Aichi virus. Virologica Sinica (under revision). Journal Impact Factor 2018: 2.46. Q2, 21/36 in Virology.



A Comprehensive Review on Human Aichi Virus

Journal:	*Virologica Sinica*
Manuscript ID	VS-2019-4626
Manuscript Type:	Review
Date Submitted by the Author:	03-Jun-2019
Complete List of Authors:	Rivadulla, Enrique; Universidade de Santiago de Compostela, Microbiology and Parasitology Romalde, Jesús L.; Universidade de Santiago de Compostela, Microbiology and Parasitology
Keywords:	Human Aichivirus, Kobuvirus, Picornaviridae, Gastroenteritis

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Significance: The aim of this review is to bring together all the discovered information about the emerging pathogen human Aichi virus (AiV), discussing the possibles routes of transmission, new detection techniques and future research. Although AiV is responsible for a low percentage of gastroenteritis outbreaks, the high seroprevalence shown by human populations indicates an important role as an enteric agent. The low percentage of AiV detection could be explained by the fact that the pathogen is more associated to subclinical infections that are mainly asymptomatic. Further studies will be needed to clarify the real impact of AiV in human health and its importance as a causative gastroenteritis agent worldwide.

Running title: A Review on Human Aichi Virus

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Summary

Although Norovirus, Rotavirus, Adenovirus and Astrovirus are considered the most important viral agents transmitted by food and water, in recent years other viruses, such as Aichi virus (AiV), have emerged as responsible for gastroenteritis outbreaks associated with different foods. AiV belongs to the genus *Kobuvirus* of the family Picornaviridae. It is a virus with icosahedral morphology that presents a single stranded RNA genome with positive sense (8,280 nucleotides) and a poly (A) chain. AiV was first detected from clinical samples and is considered today one of the main causes of nonbacterial gastroenteritis in some geographic areas. Furthermore, several studies conducted in Japan, Germany, France, Tunisia and Spain showed a high prevalence of AiV antibodies in adults (between 80 and 99%), which is indicative of a large exposure to this virus. The aim of this review is to bring together all the discovered information about this emerging enteric pathogen.

Keywords: Human Aichivirus, Kobuvirus, Picornaviridae, Gastroenteritis.

INTRODUCTION

Acute gastroenteritis is the leading cause of morbidity worldwide especially among children under 5 years old and elderly population (Patel et al., 2009). This is associated with important medical and healthcare costs. Foodborne viruses infect humans via gastrointestinal tracts and are excreted through feces, in a process known as fecal-oral route (Lee et al., 2013). Among these foodborne agents Norovirus (NoV), Rotavirus (RV), Adenovirus (AdV) and Astrovirus (AsV) are considered the most important aetiological agents of acute non-bacterial gastroenteritis, transmitted by contaminated food and water (Phan et al., 2011; Amaral et al., 2015). Although nowadays the knowledge about viral foodborne diseases is extensive, the study of fecal-oral route is relatively recent. The first documented outbreak associated with food was in 1914, the causative agent was recognized as Poliovirus and transmitted through raw milk (Jubb, 1915). This viral pathogen was later classified as a member of *Picornaviridae* family (Fenner, 1976).

HISTORY AND TAXONOMY

The picornavirus term (*pico:* small*; rna:* ribonucleic acid*)* was first introduced in 1963. This viral family includes virus of small size with single-stranded RNA (ssRNA) as genetic material and contains many important human and animal pathogens (Melnick et al., 1963; Melnick, 1996). The family *Picornaviridae* belongs to the order of Picornavirales and consists in 94 species grouped into 40 recognized genera (Figure 1) (Adams et al., 2017; King et al., 2018).

Within all of these recognized genera, this review is focused on *Kobuvirus.* It was classified as a new genus in 1999 (King et al., 1999). The

name comes from the Japanese word ´kobu´ that means bump, and it refers to the characteristic morphology of the virion that appears bumpy at the electron microscope (Yamashita et al., 2000). The first three official candidates of *Kobuvirus* were *Aichi virus*, *Bovine kobuvirus* and *Porcine kobuvirus* which were described on the basis of the host in which the viruses were detected.

Nowadays, the genus *Kobuvirus* consists in six recently renamed species: Aichivirus A (formerly *Aichi virus*), Aichivirus B (*Bovine kobuvirus*), Aichivirus C (*Porcine kobuviru*), Aichivirus D, Aichivirus E and Aichivirus F (Adams et al., 2017; Zell et al., 2017). Aichi virus A is divided in six genetically distinct groups: Aichi virus in humans (AiV) (Yamashita et al., 1991), canine kobuvirus (Kapoor et al., 2011; Barros et al, 2019), murine kobuvirus (Phan et al., 2011), Kathmandu sewage kobuvirus (Ng et al., 2012), roller kobuvirus (Pankovics et al., 2014) and feline kobuvirus (Chung et al., 2013). Along *Kobuvirus* evolution, transmission between different host taxa was reported, including a host jump from artiodactyla (sheep) to carnivora (ferret) (Bayes Factor [BF] = 62) or from carnivora to birds and humans (BF = 5) (Lu et al., 2018).

AiV was first recognized and isolated in BSC-1 (kidney of normal adult African green monkey) cells in 1989 during the study of a gastroenteritis outbreak associated with oyster consumption in Japan (Yamashita et al., 1991; 1998). Some years later, in 1998, the complete genome sequence of AiV (GenBank accession no AB040749) was described and classified as a novel picornavirus, at the Aichi Prefectural Institute of Public Health, in Japan (Yamashita et al., 2001). The first detections of AiV outside Asia were in Europe (Germany) and South America (Brazil), where AiV was detected in clinical specimens (Oh et al., 2006).

PHYSICAL CHARACTERISTICS AND STABILITY

AiV is a a small non-enveloped virus with icosahedral morphology. The virions shows a rough surface with around 30 nm in diameter at the electron microscopy and negative staining. The capsid reveals some surface depressions like "canyon" which form the site of receptor binding in many picornaviruses (Thuthill et al., 2010; Dang et al., 2014; Wang et al., 2012). *In vitro*, AiV shows stability in acid conditions as low as pH 2 and is resistant to conventional methods of inactivation, including alcohols, heat, chlorine, high hydrostatic pressure, chloroform, non-ionic detergents and ether (Yamashita et al., 1998; Cromeans et al., 2014). Even more, AiV retained infectivity after 21 days at 4ºC in Cranberry juice and Cranberry juice cocktail (pH 3.0) (Sewlikar and Souza, 2017).

GENOMIC ORGANIZATION

AiV has a ssRNA with positive sense (8,280 nucleotides), composed by a 5' untranslated region (UTR) of about 744 nucleotides with an internal ribosomal entry site (IRES) that allows direct translation of the polyprotein; an open reading frame (ORF) followed by 237 nucleotides of a 3'UTR region and a poly(A) tail (Figure 2). The ORF is about 7.3 kb and encodes a polyprotein precursor of 2432 amino acids with a non-structural leader (L) protein at the N-terminus, followed by viral capsid proteins P1 (VP0, VP3 and VP1) and, non-structural proteins P2 (2A, 2B and 2C) and P3 (3A, 3B, 3C and 3D) that control the replication of AiV in the infected cells (Sabin et al., 2016; Zhu et al., 2016).

Capsid proteins adopt an eight-stranded antiparallel beta barrel configuration with a pseudo T=3 symmetry (where T is the triangulation number) where VP0 and VP3 alternate about the two- and three-fold axes and VP1 surrounds the five-fold axes (Zhu et al., 2016) N-termini of the

major capsid proteins are located inside of the capsid, and C-termini are at the virion surface. The three capsid proteins were described with a weight of 42, 30 and 22 kDA (Yamashita et al., 1998).

VP0 capsid protein remains in the mature particles of AiV, in contrast with other picornaviruses. Also, the structural protein 2A and L protein have not shown protease or autocatalytic motifs as are detected in other picornaviruses and their functions remain unclear (Yamashita et al., 1998; Buesa and Rodriguez, 2016). On the other hand, the 2A protein contain conserved motifs that are charateistic of the H-rev107 family, which are involved in the cellular proliferation (Huges and Stanway, 2000). Attending to the other non-structural proteins, 2C, 3C and 3D regions are well aligned with the corresponding sequences of other picornavirus as *Aphthovirus*, *Cardiovirus*, *Parechovirus.* The 3B protein (VPg) of AiV is longer than other viruses of the same family, and the 3C is the protease which contains conserved motifs characteristic of all picornaviruses (Yamashita and Sakae, 2003). Furthermore, a complex formed with 2B, 2BC, 2C, 3A, and 3AB proteins, Golgi apparatus protein ACBD3 and phosphatidylinositol 4-kinase IIIβ (PI4KB) at viral RNA replication sites, enhancing PI4KB-dependent phosphatidylinositol 4-phosphate (PI4P) production, is crucial for AiV replication (McPhail et al, 2017; Ishikawa-Sasaki et al, 2018; Klima et al., 2019)

VIRAL REPLICATION

As other *Picornaviridae* members, AiV enter their host cells by receptor-mediated endocytosis. After adsorption, the virus penetrates the cell through little known mechanisms, that followed the stripping of virions and the release of viral RNA into the cytoplasm. Using ribosomes and other

cellular proteins, the viral RNA (which is already mRNA), forms polyribosomes for the direct synthesis of a polyprotein.

The polyprotein is co- and post-trasnlationally processed into the L protein, capsid proteins, mature nonstructural proteins and stable intermediates. These proteins modify the cellular environment and promote synthesis of RNA (-) required for the complement RNA (+). When the protein combination increases, also increases the number of RNA (+) in the replicative complex, that will be encapsidated after joining VPg (Belov, 2016, van der Scharr et al, 2016).

As a preliminary step in the assembly, one of the the cover precursors (P1) is cut by viral proteinases to form a 5S subunit (immature promoter) composed of three protein aggregates (VPO, VP2 and VPl). The 5S subunit forms pentamers, 12 of which they are required to form the 60 protein subunits of the envelope.

Once the maturation process has finished, the complete viral particles has 60 copies of each capsid protein, one copy of the ARN (+) genome and one copy of VPg; these often form crystals in the cytoplasm and are finally expelled to the outside by the lysis of the infected cell. (Zell, 2017).

MOLECULAR CHARACTERIZATION AND DISTRIBUTION

Genetic Diversity

Although typing VP1 sequence is a suitable method for picornavirus classification because is diversified region (Oberste et al., 1999), 3CD junction is also used for that purpose too because the high correlation of AiV genotypes at this region (Yamashita et al., 2000; Ambert-Balay et al., 2008; Pham et al., 2010),

The first genetic differentiation of AiV genotypes was made by Yamashita and coworkers (2000). In that study the genetic relation among 17 AiV isolates was predicted by the comparison of 519 bases at the putative junction between the C terminus of 3C and the N terminus of 3D, and two groups were proposed: Group 1 or genotype A and group 2 or genotype B. Since then, 3 distinct genotype categories A, B and C were described (Kitajima and Gerba, 2015). Furthermore, several studies suggest some geographical distribution of AiV genotypes (Figure 3).

Genotype A

Among human population, the presence of genotype A has essentially been reported in Asian countries. As said before, AiV A was detected in samples connected with foodborne outbreaks in Japan (Yamashita el al., 2000). In addition, AiV A was detected in stool samples from patients with acute gastroenteritis from Indonesia, Bangladesh, Vietnam and Thailand (Yamashita and Sakae, 2003, Pham et al., 2007, Chuchaona et al., 2017).

In Europe the presence of AiV A was reported for the first time in Germany, from stool samples of patients involved in a gastroenteritis outbreak (Oh et al., 2006). Afterwards the same genotype was found in France, in samples from children and adults hospitalized for acute gastrointestinal illness (Ambert-Balay et al., 2008), in Finland from children under 5 years with gastroenteritis (Kaikkonen et al., 2010) and in a Hungarian sample from a 3 years old girl (Reuter et al., 2009). AiV A was also reported in elderly patients suffering from diarrhea, who were negative for AdV, RV, and calicivirus in Sweden (Jonsson et al., 2012) in Italy (Bergallo et al., 2017) and among different age groups from outpatients in Spain (Rivadulla et al., unpublished results).

Finally, AiV A was also reported in Africa. It was detected in stool samples from inpatient and outpatient children collected in Monastir, Tunisia (Sdiri-Loulizi et al., 2009).

Genotype B

AiV B was described at the same time as AiV A in samples from Japan and Malaysia, but with less prevalence (Yamashita et al., 2000). Although genotype A seems to be predominant in Asian population, there are some Asian locations where the presence of AiV B is more prevalent. China, Pakistan and Bangladesh are part of this exception and AiV B has been detected more often than other genotypes (Pham et al., 2007; Yang et al., 2009, Li et al., 2017). On the other hand, in South Korea genotype prevalence is not clearly detected, since both genotypes were detected equally in stool samples from teenagers and adults (Han et al., 2014). In other places as Thailand, alternance of AiV A and AiV B as predominant genotypes along the time was observed (Yamashita et al., 2000; Saikruang et al. 2014; Chuchaona et al., 2017).

In Europe, AiV B was also reported. In Finland and Spain AiV B was detected, but at lower prevalence than AiV A (Kaikkonen et al., 2010; Rivadulla et al., unublished results). In Germany a genotype drift was detected, and AiV B seems to be more prevalent than in previous surveys (Drexler et al., 2011).

Finally, AiV B was detected in stool samples from Brazilian children suffering diarrhea (Oh et al., 2006), and in North American children between 15 days and 5 years of age with symptoms of acute gastroenteritis (Chhabra et al., 2013). In Africa, AiV B was detected from one outpatient children in Nigeria (Japhet et al., 2019)

Genotype C

Only one survey reported AiV C from a stool sample of a child, hospitalized for gastroenteritis in France after a trip to Mali (Ambert-Balay, 2008; Reuter et al., 2011). Further studies are needed to clarify the real impact of this genotype in human health.

LABORATORY DIAGNOSIS

Detection Methods

Since AiV was discovered various methods have been used to identify it. Although AiV have not shown cytopathic effect (CPE) in human cell lines as HeLa (Human cervix epitheloid carcinoma), HEL (Human Erythroleukemia Cell Line), RD (Human rhabdomyosarcoma Cell Line) cells or in newborn mice, AiV cause CPE on BSC-1 and Vero (both obtained from kidney of African green monkeys) cells. (Yamashita et al., 1993). However, cell detection method is time consuming and is less efficient than other methods for AiV detection (Yamashita et al., 2000; Kitajima and Gerba, 2015).

Another method for detection is electron microscopy. The viral particles have a distinct ultrastructure than other gastroenteritis pathogens as SaV or NoV, but there are not easy to distinguish from other small round viruses and could be wrongly be classified as an AsV (Yamashita et al., 1993).

An enzyme-linked immunosorbent assay (ELISA) was also developed for the detection of AiV antigens in clinical samplesusing monoclonal antibodies (Yamashita et al., 1993). Although ELISA is better than culture isolation for AiV detection, it is also time consuming and less sensitivity

than other methods (Yamashita et al., 2000). Furthermore, it is of limited value for detection of antigenetically diverse strains.

Reverse transcription-polymerase chain reaction (RT-PCR) is a widely employed method for AiV research. Is a sensitive method applicable for further genetic analysis as genotyping (Yamashita et al., 2000). As mentioned before, the 3CD junction region is suitable for differentiation of AiV genotypes, but VP1 region is also used for the same purpose. Nonetheless, no phylogenetic evidence of recombination has been reported between AiV genotypes A and B, suggesting that both genotyping methods can result in the same genotype (Lukashev et al., 2012; Kitajima and Gerba, 2015). Nested PCR targeting the 3C, VP1, and VP3 regions, as well as multiplex semi-nested PCR on VP0-VP3 region were also developed for genotyping (Oh et al., 2006; Lodder et al., 2013).

Recently, Oshiki et al. (2018) reported a high-throughput detection and genotyping tool for RNA virus, like AiV, using a microfluidic device and next-generation sequencer. On this study the investigators reported detection limits ranging from 10^0 to 10^3 copies/μL in cDNA sample, corresponding to 10^1-10^4 copies/mL-sewage, 10^5-10^8 copies/g-human feces, and 10^2-10^5 copies/g-digestive tissues of oyster. Simultaneous detection and genotyping techniques are a powerful tool for source tracking of human pathogenic viruses.

Quantification Methods

Although conventional culture methods like 50% Tissue culture Infective Dose ($TCID_{50}$) are used to quantify AiV, methodological advances in molecular biology lead to the development of better technologies. Reverse transcription- quantitative PCR (RT-qPCR) is nowadays the most employed method for AiV detection and quantification. It has too many advantages

than conventional methods including higher specificity, lower contamination risk or capability of multiplex strain reaction using multiple probes with different reporter dyes (Kitajima and Gerba, 2015). The highly conserved 5´-UTR sequence is a common RT-qPCR target for picornavirus detection (Drexler et al., 2011, Nielsen et al., 2013). However, a RT-qPCR that amplifies the VP0 region, and could quantify and differentiate between genotypes A and B, was developed for determination of viral RNA load in clinical and environmental samples (Kitajima et al., 2013). This RT-qPCR consists in two amplifications: one that uses a universal primer pair that could amplify both genotypes and a universal probe to detect AiV. The second one uses the same primer pair but two different genotype-specific probes. Both methods, VP0 qPCR and 5´-UTR qPCR showed similar efficiency for AiV detection, with the advantage that VP0 qPCR is able to quantify and also differentiate the AiV genotypes.

Although, these techniques have been helpful to clarify the transmission sources of AiV among population, during the recent years, new detection technologies have been developed. Digital RT-PCR (RT-dPCR) is a precise endpoint-sensitive absolute quantification approach, capable of determine the number of target copies without a standard curve. As an example, AiV and eighteen enteric viruses more were targeted with this method and compared with RT-qPCR (Coudray-Meunier et al., 2016), the conclusions were that the limit of detection for RT-dPCR array assays was lower (by 0.8 to 3.8 $\log^{10}$) than the limits of detection obtained with conventional RT-qPCR. This new technology presents many advantages and possibilities for detection of enteric pathogens in environmental and clinical samples. The RT-dPCR divides each reaction mix across thousands of individual PCR reactions, making this method more tolerant to inhibitory substances and also reducing the difficulty of virus quantification (Pinheiro et al., 2012; Rački et al., 2014).

CLINICAL AND EPIDEMIOLOGICAL OBSERVATIONS

Symptoms and importance of Disease

Gastrointestinal illness is commonly associated with symptoms produced by enteric viruses but usually masked for the presence of other pathogens (Yamashita and Sakae, 2003). AiV replicate in the gastrointestinal tract, resulting usually in symptomless infections. Diarrhea, abdominal pain, nausea, vomiting and fever are the clinical symptoms but pathogenesis of AiV releases more in subclinical infections than in clinically manifest diseases (Yamashita et al., 1991; 2001). These subclinical infections do not need healthcare attention and underestimate the real impact of AiV in human health (Bergallo et al., 2017).

Nowadays, the clinical role of AiV as gastrointestinal pathogen stills unclear. Some surveys support the idea that AiV produce the outbreaks in coinfection with other viruses, because its frequent codetection with other enteric pathogens. This idea is also supported for the low incidence rates (0.9%-4.1%) of AiV outbreaks (Kaikkonen et al., 2010; Jonsson et al., 2012; Nielsen et al., 2013). However, other studies showed that the coinfection of AiV with other enteric viruses occurs at low percentage (Yang et al., 2009) and is not present in healthy individuals, supporting the idea of AiV as an important gastrointestinal pathogen (Drexler et al., 2011, Chhabra et al., 2013).

When the illness appears is generally mild, lasting 48-72h as is usual in this kind of enteric viruses (Carter, 2005). AiV replicate and destroy the enterocyte layer that cover the upper third of intestinal villi. That destruction of functional and mature cells interrupts the reabsorption of water and diarrhea ensues. As a response, the villi gets retracted and the absorption surface area decreases. Meanwhile, crypt cells undergo rapid division to

repopulate the villi with immature cells resistant to the infection, but the younger cells can not replace the function of those that were infected because they require time to mature and diarrhea appears, sometimes with fatality results (Carter, 2005).

AiV is a cause of chronic infection in X-linked agammaglobulinemia and its prevalence in stool samples from patients with human immunodeficiency virus is high, indicating an opportunistic behavior in people with underlaying T cell defects (Oude et al., 2014; Portes et al., 2015; Bucciol et al., 2018). A Hungarian case was also remarkable (Reuter et al. 2009). A 3 years old girl that had rhinitis, purulent conjunctivitis and diarrhea was positive for *Kobuvirus*. Also, after the sampling day she developed bronchopneumonia and fever with an elevated erythrocyte sedimentation rate.

Immunity

Virological surveys suggest that AiV is responsible for a low percentage (0.5%-1.8%) of gastroenteritis sporadic cases (Kitajima et al., 2015; Alcalá et al., 2018). However, high prevalence of antibodies against AiV (anti-AiV) was observed over the world.

The first seroprevalence study was carried out in Japan. In that survey the highest rate of anti-AiV was observed in adults of about 35 years old (Yamashita et al., 1993). Since then, several studies detected high levels of AiV seroprevalence worldwide and its correlation with the patients age.

Several studies reported high percentages of AiV seroprevalence in Europe. In Germany, the percentages rise from 51%, in children aged from 0 to 3 years, to almost 100%, in patients >40 years old (Oh et al., 2006). Similar results were achieved in France, where these percentages increased steadily from 25% in infants between 7 months to 9 years old to 85–90% in

patients older than 40 years, with no significant variations among age groups after age 40 (Goyer et al. 2008). In Spain, a significant increase of anti-AiV was observed up to the age of 30 years old, with the absence of a significant increase after that age (Ribes et al. 2010). Also, in the same study the percentage of anti-AiV observed was 100% in individuals over the age of 40 years old.

Serological studies carried out in Tunisia, revealed also a high prevalence of anti-AiV in humans of different age groups (Sdiri-Loulizi et al., 2009). As in the previous studies, the presence of anti-AiV throughout population increased with age raising from 68.8%, in patients between 6 months and 10 years old, to 100% in elderly patients between 71 to 89 years old. This same study showed no significant variations in seroprevalence in patients >30 years old.

Considering all of these data, it is possible to conclude that the seroconversion to AiV occurs during childhood or adolescence indicating an important role of AiV as a causative agent of pediatric diarrhea (Sdiri-Loulizi et al., 2009). Also, the high level of seroprevalence in adults showed in these surveys suggest a widespread exposure among human population to AiV.

ENVIROMENTAL OCCURRENCE

AIV in shellfish

Bivalve molluscs are associated with viral foodborne disease (Vossen, 2001) as they obtain their food filtering small particles suspended in water. Often in these processes, molluscs concentrate and retain pathogens including enteric viruses (Romalde et al, 1994). These viruses are underestimated in molluscan safety controls that are based only on bacterial

indicators, becoming this kind of food as a vector for enteric viruses transmission (Polo et al., 2015).

From its first detection, AiV has been suggested as an important etiological agent of gastroenteritis especially in outbreaks associated with contaminated seafood (Table 1) (Yamashita et al., 1991). In Japan, a one-year study carried out between 2005 and 2006, reported AiV A from clam samples, with a prevalence of 73% (Hansman et al., 2008).

More studies reported AiV from shellfish samples worldwide. In Tunisia, Sdiri-Loulizi et al. (2010) reported an AiV prevalence of 4% in shellfish. Aditionally, the phylogenetic analysis revealed several clusters that occurred sequentially in time, pointing out some parallelism in the temporal shifts among environmental and human strains. On the other hand, Onosi et al. (2019) observed prevalences up to 66.6% in mussels from South Africa.

In France, other study detected AiV in oysters that were linked to a gastroenteritis outbreak and the AiV sequences obtained where similar to those from stool samples analyzed in parallel (Le Guyader et al., 2008). More recently, AiV has been detected shellfish from Spain and Italy (Fusco et al., 2017; Rivadulla et al, 2017; Terio et al., 2018), with prevalences ranging from 1.7 to 12%.

Wastewater

AiV was identified in water in 2010 (Alcalá et al., 2010). Since then, AiV was reported in high percentages in wastewater samples around the world (Table 2). Current treatments (when present) applied in wastewater treatment plants (WWTPs), can not guarantee a total removal of viral pathogens, that are continuously discharged to the environment (da Silva et al, 2007).

In Japan AiV was detected in high percentages (from 66.2% to 100%) in raw sewage samples, with viral concentrations ranging from 1.4 x 10^5 to 2.2 x 10^7 copies/L (Yamashita el al., 2014; Kitajima et al., 2011, 2013). Samples of treated sewage also analyzed showed an AiV prevalence of 91.7% (Kitajima et al., 2011). AiV detection in sewage showed no seasonality, being detected throughout the year (Thongprachum et al., 2018). In other Asian countries, as Nepal or Thailand, AiV was also reported from untreated sewage samples (Ng et al., 2012; Haramoto and Kitajima, 2017). These studies supported the idea of use AiV as a human faecal pollution indicator, due its stability in wastewater and his lower removal percentages during wastewater treatments.

Other surveys detected AiV in Africa, America and Europe. In Africa, AiV was detected in Tunisia but at low prevalences. Thus, Sdiri-Loulizi et al. (2010) detected AiV in 10 out of 125 (8%) samples of influent water samples and 4 out of 125 (3.2%) treated sewage samples. On the other hand, Ibrahim et al. (2017) reported the virus in 51 out of 102 (50%) samples. Moreover, a recent study carried out in South Africa detected AiV in 10 out of 12 pooled sewage samples (Onosi et al., 2019).

In America, AiV was detected in a sample of untreated wastewater collected from Pennsylvania (Cantalupo et al., 2011). Furthermore, another study carried out in two WWTPs of southern Arizona, detected AiV in all influent and effluent samples, with viral levels of 1.2 x 10^4 to 4.0 x 10^6 copies/L in influent samples and 2.0 x 10^3 - 4.0 x 10^5 copies/L in effluent samples (Kitajima et al, 2014). A clear predominance of AiV B was revelaed in these positive samples (Kitajima et al., 2018).

Finally, in Europe the percentages of detection vary. Thus, AiV was observed in sewage samples from France, the Netherlands and Spain with detection rates between 61 and 100% (Cantalupo et al., 2011; Lodder et al., 2013; Prevost et al., 2015). However, other analysis of untreated influent

sewage samples collected from four WWTPs in Italy detected AiV in only 12.5% of the samples (Di Martino et al., 2013).

River water and Groundwater

Surface water is infiltrated via spreading basins into aquifers and wells but, due to their small size and survival capacity, viral pathogens like AiV are not totally removed duing this natural filtration (Weiss 2005; Sharma and Amy, 2010). Therefore, river water and groundwater are also possible reservoirs for AiV (Table 3).

Although AiV had been previously detected in tap water in America (Rosario et al., 2009), the first study conducted to determine the occurrence and circulation of AiV in river water was carried out in Venezuela. In this study, AiV was detected in 5 out of 11 samples (45%) (Alcalá et al., 2010). Other survey carried out in Japan for a longer period detected AiV in 36 out of 60 samples (60%), demonstrating a higher detection frequency for AiV than for other enteric virus, like NoV or SaV is the same set of river samples (Kitajima et al., 2011). Hata et al. (2014), investigating the effects of rainfall events and water quality on viral occurrence, detected AiV in all the tested samples with relatively higher frequency of detection and concentration (ranging from 1.2 x 10^6 to 8.3 x 10^8 copies/L) than other enteric viruses. More recently, the same authors detected AiV in 20 out of 50 surface water samples in Japan (Hata et al., 2018).

In Nepal, AiV was detected from river water, groundwater, tap water in a house supplied by tanker water, and from a sewage pipe (Haramoto and Kitajima, 2017). In this study, differences in AiV detection were observed, and a high prevalence of AiV B was reported. The frequence of AiV detection was significantly higher in shallow dug wells, where AiV was found in 10 out of 22 samples (45%) than in shallow tube wells, in which

AiV was found in 1 out of 15 samples (7%). In accordance with the study, this could be happened due the vulnerable structure of dug wells, which are usually made of brick or stone, than tube wells.

The first study for AiV detection in groundwater was performed in USA for the assessment of the occurrence and elimination of virus at a full-scale managed aquifer recharge system (Betancourt et al., 2014). In this study, the concentration of AiV was up to 1.52×10^4 copies/L. Recent environmental studies have demonstrated a high prevalence of AiV in different types of water samples, such as river and groundwater (Kitajima and Gerba 2015).

There are also some surveys that reported AiV from river samples in Europe. In France 20 out of 175 river water samples were positive for AiV with up to 10^2 copies/L concentration levels (Prevost et al., 2015). In the Netherlands, AiV was also detected in 12 out of 14 river water samples (Lodder et al., 2013). The importance of that pathogen in this kind of samples is not well understood and more investigation will be needed to evaluate the real impact of AiV.

CONCLUDING REMARKS

Since the first documented outbreak associated with food it has become evident the importance of viruses as pathogens that cause alimentary illness. Also, the ineffectiveness of the current mechanisms of microbiological control in food, from a virological point of view, as well as the current vulnerability of our global food market are key factors in the appearance of outbreaks (Polo et al., 2010).

AiV has become a risk to public health as an etiological agent of gastroenteritis. Due its stability in the environment, AiV virions could stay for long periods present in different matrices as sewage, water and foods.

Also, the low infection dose that is needed for AiV infection makes possible its continuous circulation, allowing the geographical spread of AiV genotypes.

Although AiV is responsible for a low percentage of gastroenteritis outbreaks, the high seroprevalence shown by human populations indicates an important role as an enteric agent. The low percentage of AiV detection could be explained by the fact that the pathogen is more associated to subclinical infections that are mainly asymptomatic. However, the methodological advances in molecular biology leads to the development of new technologies that improve the detection of these pathogens. Furthermore, these new methodologies are also lighting the investigation to clarify the transmission routes of AiV infection, solving the limitations of the classical detection methods. Further studies will be needed to clarify the real impact of AiV in human health and its importance as a causative gastroenteritis agent worldwide.

ACKNOWLEDGMENTS

Prof. Jesús L. Romalde acknowledges the support of Consellería de Educación, Universidade e Formación Profesional, Xunta de Galicia, Spain (Grant 2014-PG110), that funded the research that generated part of the results presented here.

CONFLICT OF INTEREST

The authors declare no conflict of interest

COMPLIANCE WITH ETHICS GUIDELINES

This article does not contain any studies with human or animal subjects performed by any of the authors.

AUTHORS' CONTRIBUTION

Both authors contributed to the writing and revision of the manuscript and approved the submitted version.

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752 **Table 1.** Worldwide detection of AiV in shellfish. NS, not specified; NQ, non quantifiable (under the limit of quantification of the method).

753

Country	Positive Samples	Sample type	Genotype	copies/L	Reference
South Africa	8/12	Mussels	NS	NS	Onosi et al., 2019
Tunisia	4/60	Shellfish (NS)	A	NS	Sdiri-Loulizi et al. 2010
Japan	19/26	Clams	A	NS	Hansman et al., 2008
France	6/66	Oysters	NS	NS	Le guyader et al. 2008
Italy	13/108	Mussels	NS	up to 10^2	Fusco et al. 2017
	3/170	Mussels, Oysters, Clams	A,B	NS	Terio et al., 2018
Spain	15/249	Mussels, Clams, Cockles	NS	NQ - $6{\cdot}9 \times 10^3$	Rivadulla et al., 2017

754

756 **Table 2.** Worldwide detection of AiV in Wastewater. NS, not specified.

757

Country	Positive Samples	Sample type	Genotype	copies/L	Reference
South Africa	10/12	Raw sewage	NS	NS	Onosi et al., 2018
Tunisia	10/125	Raw sewage	A	NS	Sdiri-Loulizi et al., 2010
	4/125	Treated sewage	A	NS	Sdiri-Loulizi et al., 2010
	51/102	Raw sewage	B	NS	Ibrahim et al., 2017
US	24/24	Raw sewage	A,B	1.2×10^4 - 4.0×10^6	Kitajima et al., 2014, 2018
	24/24	Treated sewage	A,B	2.0×10^3 - 4.0×10^5	Kitajima et al., 2014, 2018
	1/1	Raw sewage	NS	NS	Cantalupo et al., 2011
Japan	137/207	Raw sewage	A	NS	Yamashita et al., 2014
	12/12	Raw sewage	A,B	NS	Kitajima et al., 2011
	11/12	Treated sewage	A	NS	Kitajima et al., 2011
	12/12	Raw sewage	A	1.4×10^5 - 2.2×10^7	Kitajima et al., 2013
	11/12	Treated sewage	A	Up to 1.8×10^4	Kitajima et al., 2013
	11/12	Raw sewage	NS	NS	Thongprachum et al., 2018
Nepal	1/1	Raw sewage	NS	NS	Ng et al., 2012
	1/1	Raw sewage	B	NS	Haramoto and Kitajima, 2017
Thailand	1/1	Raw sewage	NS	NS	Ng et al., 2012
France	61/100	Treated sewage	NS	Up to 10^3	Prevost et al., 2015
Italy	6/48	Raw sewage	B	NS	Di Martino et al., 2013
Netherlands	16/16	Raw sewage	A,B	NS	Lodder et al., 2013
Spain	1/1	Raw sewage	NS	NS	Cantalupo et al., 2011

758

760 **Table 3.** Worldwide detection of AiV in water. NS, not specified.

761

Country	Positive Samples	Sample type	Genotype	copies/L	Reference
US	1/2	Reclaimed water	NS	NS	Rosario et al., 2009
Venezuela	5/11	River water	B	NS	Alcalá et al., 2010
Japan	36/60	River water	A+B	NS	Kitajima et al., 2011
	29/29	River water	NS	8.6×10^2 - 2.0×10^4	Hata et al., 2014
	20/52	Surface water	NS	Up to 10^4	Hata et al., 2018
Nepal	14/14	River water	B	1.2×10^6 - 8.3×10^8	Haramoto and Kitajima, 2017
	1/1	Tap water	B	10^9	Haramoto and Kitajima, 2017
France	20/175	River water	NS	Up to 102	Prevost et al., 2015
The Netherlands	12/14	River water	A+B	NS	Lodder et al., 2013

762

767 **Figure Legends**

768
769 **Figure 1.** Phylogenetic tree, based on the P1 protein gene, showing the relationships among the
770 classified and unclassified members of the family *Picornaviridae.* The maximum likelihood tree
771 was constructed using MEGA 7.6. GenBank accession numbers of the reference strains used are
772 detailed in the tree.

773

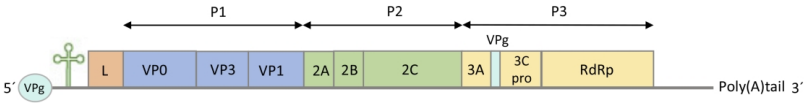
774 **Figure 2.** Schematic of the monopartite, linear, ssRNA(+) genome of AiV.

775

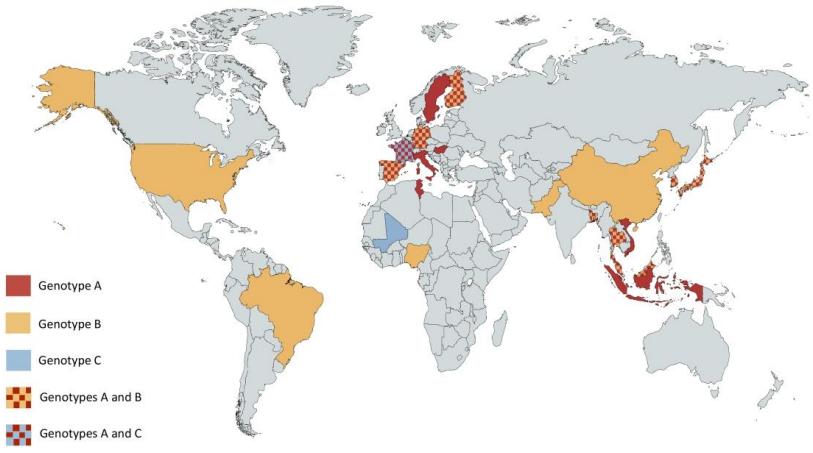
776 **Figure 3.** Worldwide distribution of AiV genotypes.

777

Phylogenetic tree, based on the P1 protein gene, showing the relationships among the classified and unclassified members of the family Picornaviridae. The maximum likelihood tree was constructed using MEGA 7.6. GenBank accession numbers of the reference strains used are detailed in the tree.



Schematic of the monopartite, linear, ssRNA(+) genome of AiV.



Worldwide distribution of AiV genotypes.

1.4 Hepatitis E virus.

1.4.1 History and taxonomy.

In 1978 an epidemic non-A non-B hepatitis outbreak occurred in Kashmir (India) affecting two hundred villages and caused around 52,000 patients with icteric disease and 1,700 fatalities (Khuroo, 1991, 2011). On that outbreak, virions were found to lack markers of acute hepatitis A or B and had triggered repeated epidemics in this area.

Five years later, another non-A, non-B hepatitis outbreak was reported among Russian military personal that were in Afghanistan. On that case, thanks to a hazardous self-experiment carried out by Mikhail S. Balayan, the disease was deeper studied (Balayan et al., 1983). The doctor ingested pooled stool extracts belonging to 9 patients and developed a severe hepatitis with jaundice and elevated liver tests. During the illness time, the doctor collected his own stool samples that showed spherical virus-like particles at the ME. The pathogen was inoculated in monkeys for physicochemical properties study and was identified as HEV (Balayan et al., 1983; Bradley et al., 1991).

For a long period HEV was only reported from humans and restricted to areas with poor water supply and sanitation. This vision was enhanced with the endemicity of HEV in developing countries and its correlation in the developed countries with travelers from those areas (Wu et al., 1998; Schwartz et al., 1999). Nonetheless, in the 90s some cases of HEV infection were reported among residents from developed countries that had not traveled to countries where HEV was

endemic (Kwo et al., 1997; Erker et al., 1999), and genomic sequences of HEV-like virions were identified in wild and domestic animals (mainly pigs) (Meng et al., 1997). Nowadays, we know that HEV shows a high degree of genomic heterogeneity as well as a wide host range. Also, many surveys reported cross-species transmission and its worldwide circulation (Aggarwal, 2013; Forni et al., 2018).

Originally HEV was included in the *Picornaviridae* family (Sreenivasan et al., 1984), but when morphological features and genome organization were studied, HEV was placed on *Calciviridae* family due its similarities with NoV, under the *Hepevirus* genus. Time after, HEV was removed from this family and included in "Hepatitis E-like virus" because the significant sequence differences (Emerson et al., 2005a). Nowadays, according to phylogenetical features and hosts, HEV was placed in the *Hepeviridae* family (Meng et al., 2012). The family includes two genera: *Orthohepevirus,* divided in 4 groups (*Orthohepevirus A-D*), which affect mammalian and avian species, and *Piscihepevirus*, consisting only in one group that infect trouts (Smith et al., 2014).

HEV strains infecting humans belong to the *Orthohepevirus* A species, which is composed by 8 genotypes (HEV-1 to HEV-8) (Figure 8). Genotypes HEV-1 and HEV-2 only infect humans, whereas HEV-3 and HEV-4 also affect domestic and wild animals. Genotypes HEV-5 and HEV-6 affects wild boars (Forni et al., 2018) and genotype HEV-7 has been detected recently in dromedary camels (Lee et al 2016; Smith et al. 2013). In addition, this same genotype was also reported from a immunocompromised person who consumed

regularly, dromedary goods (Lee et al., 2016). Finally, genotype HEV-8 was isolated from Bactrian camels (Woo et al., 2016). The genotypes HEV-8 and HEV-5 were experimentally transmitted to macaques, demonstrating the possibility of zoonotic infections (Li et al., 2019; Wang et al., 2019).

1.4.2 Genetic structure and physicochemical stability.

HEV is a non-enveloped, positive-sense, ssRNA virus with an icosahedral capsid and a diameter between 27 to 34 nm (Balayan et al. 1990; Reyes et al. 1991). RNA genome is about 7,2 Kb composed by three discontinuous and partially overlapping open reading frames (ORF) (Meng et al. 2012). ORF1 encodes a polyprotein with functional domains present in other positive-strand RNA viruses. ORF2 encodes a capsid protein responsibly of the virion assembly. ORF 3 encodes a small protein involved in virion morphogenesis and release (Figure 9) (Kamar et al. 2015).

ORF1 is approximately 5 kb in length and can be translated directly from the viral genome to produce a non-structural protein having 1693 amino acids (Emerson et al., 2005a; Guu et al., 2009). It seems to contain several consensus sequences, such a methyltransferase, a Y domain, papain-like cysteine protease, a proline-rich hinge domain V, a X macrodomain associated with nucleoside triphosphate (NTP)-binding, an RNA helicase and RNA-RdRp needed to replicate the genomic RNA (Guu et al., 2009; Khuroo et al., 2016).

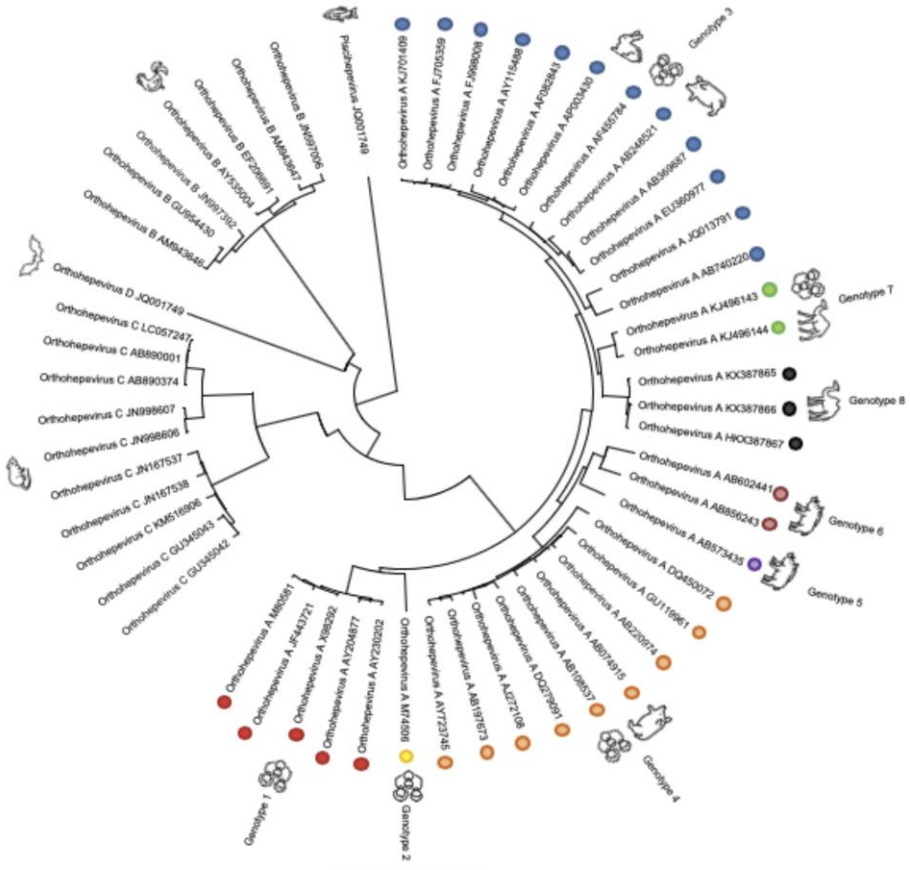


Figure 8. Phylogenetic trees for members of the *Hepeviridae* family. Maximum likelihood trees for amino acid distances were created using MEGA 6 (Tamura et al., 2013). 1000 bootstrap replicates were used. ORF1 sequences from *Hepeviridae* family were used with regions between 485-776 aa and 923-931 aa removed from the analysis due to indels within these regions. *Orthohepevirus* C are also able to infect errets, mink, Asian musk shrew, and greater bandicoot (Smith et al. 2014). *Orthohepevirus* B were also deteted in turkeys, little egrets (Reuter et al. 2016), and wild birds (Zhang et al. 2017).

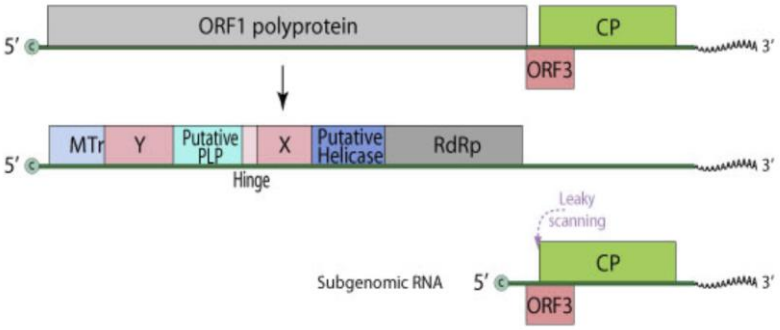


Figure 9. Monopartite linear ssRNA(+) genome of HEV. ORF1 contain several consensus sequences, such a methyltransferase, a Y domain, papain-like cysteine protease, a proline-rich hinge domain V, a X macrodomain associated with nucleoside triphosphate (NTP)-binding, a RNA helicase and RNA-RdRp. Recombinant HEV polymerase bind to the genomic RNA at 3' end. The stem-loop structures and poly (A) stretch facilitate the virion binding. The ORF2 is approximately 2 kb in size and encodes a capsid protein involved on virus assembly and binding. Overlapping both ORF1 and ORF2 a third ORF3 is situated.

ORF2 is approximately 2 kb in size and encodes a capsid protein involved on virus assembly and binding, which is also responsible of the immune response to produce neutralizing antibodies. ORF3 is situated overlapping both ORF1 and ORF2. It is translated from bicistronic subgenomic RNA and is multifunctional. Furthermore, this protein encodes a phosphoprotein that is cytoskeleton-associated and is also implicated in hepatocytes egression, is composed by two highly hydrophobic domains at its

amino terminus, D1 and D2, two proline-rich domains (P1 and P2) toward the carboxyl terminus. (Zafrullah et al., 1997).

An ORF4 has also been reported in genotype HEV-1 (Nair et al., 2016). It is overlapping the X macrodomain and the helicase domain of the ORF1. ORF4 is controlled by an internal ribosome entry site-like RNA structure that directs translation of ORF4 under endoplasmic reticulum stress. The ORF4 encodes a protein that seems to stimulate viral RdRp activity improving the viral replication (Kenney and Meng, 2018).

Attending to physicochemical stability, the virion is unaffected by chloroform and ether treatments and is stable in acid and mild alkaline conditions. Also, it has been reported that the virions remain infective in pig liver homogenates after an incubation of 1h at 56ºC, but are completely inactivated by boiling or frying for 5 min. Chlorine disinfection also affects HEV and a 1000-fold infectivity reduction has been reported after treatment of liver suspensions with Tween-20 (0.05%) and formalin (0.05%) (Emerson et al., 2005b; El-Senousy et al., 2014; Girones et al, 2014).

1.4.3 Viral replication.

Nonenveloped HEV virions attach to cellular receptors (Figure 10). Heparan sulfate proteoglycans (HSPGs) and heat shock cognate protein 70 (HSC70) are thought to be the binding receptors. After that, the virions enter into the cells through dynamin-2, clathrin, membrane cholesterol, and actin dependent endocytosis (Holla et al., 2015; Kalia

et al., 2009). Quasienveloped virions are capable to enter the cell due a clathrin-mediated pathway. This way of entry seems to be dependent on Rab5 and Rab7 GTPases and requires afterward a lysosome function before uncoating within the host cell. The transport of HEV virions to genome release sites, is mediated by heat shock proteins including HSP90 and Grp78. Once released into the cytoplasm, HEV utilizes the host translation machinery to translate the ORF1 polyproteins that include viral enzymes. This translation occurs at the rough endoplasmic reticulum (RER) as well as RER-derived membrane vesicles. ORF2 and ORF3 encoded proteins are also translated from the viral subgenomic RNA (Debing et al., 2014).

The assembly of RNA and ORF2 protein forms the next generations of virions. The interaction between conserved PSAP motifs of ORF3 protein and the host vacuolar sorting proteins like tumor susceptibility gene 101 is essential for the maturation and egression of HEV (Nagashima et al., 2011a, 2011b). Host cell membrane serves as release site of enveloped virions by exosomal release pathway. After that the viral envelope is lost. However, HEV virions released into the bloodstream seems to retain the quasienvelope, which looks to mask the capsid shell from the host immune system (Kenney and Meng, 2018).

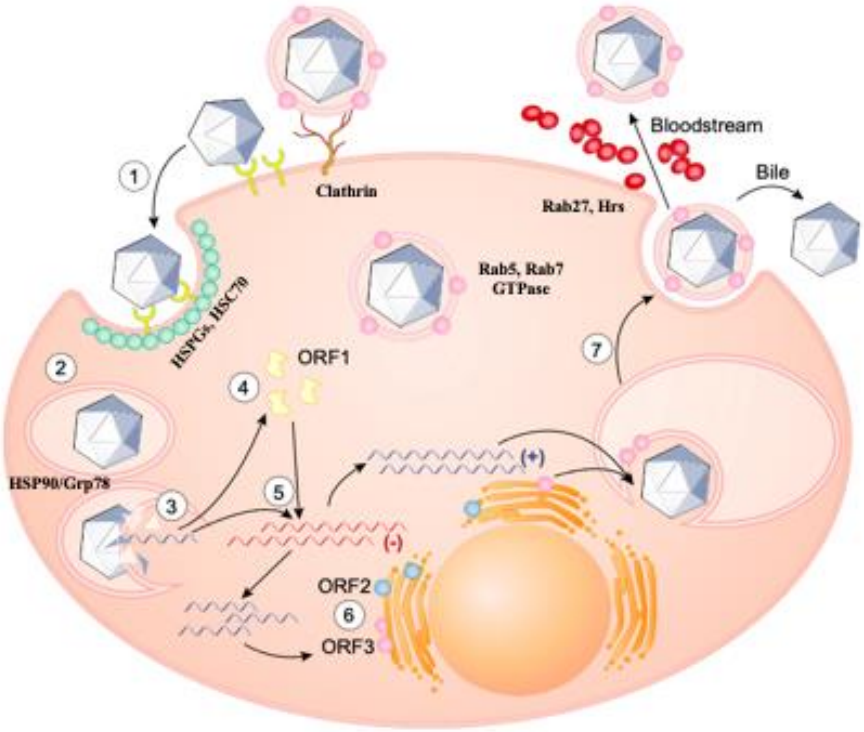


Figure 10. Viral replication of HEV virions.HEV. HSPGs and HSC70 are thought to be the binding receptors. After that, the virions enter into the cells through dynamin-2, clathrin, membrane cholesterol and actin dependent endocytosis. Quasienveloped virions enter the cell due a clathrin-mediated pathway dependent on Rab 5 and Rab7 GTPases. The transport of HEV virions to genome release sites is mediated by heat shock proteins including HSP90 and Grp78. Once into the cytoplasm HEV utilizes the host translation machinery to translate the ORF1 polyproteins. This translation occurs at RER as well as RER-derived membrane vesicles involving host proteins, such elongation initiation factors or viral proteins. ORF2 and ORF3 proteins are also translated from the viral subgenomic RNA (Image modified from Debing et al., 2016).

1.4.4 Genetic diversity.

As mentioned before, there are 4 main HEV genotypes (HEV-1 to HEV-4) that infect humans. HEV-1 and HEV-2 exclusively infect humans, whereas HEV-3 and HEV-4 seem to be mainly porcine genotypes, although they were found in other mammalian reservoirs (deer and mongooses). Since its identification, HEV has been reclassified many times, therefore it is difficult to trace the evolutionary ancestry of HEV. HEV-1 and HEV-2 are anthropotropic variants (only identified in humans) and differ from HEV-3 and HEV-4 enzooitic variants (recognized as zoonotic pathogens) in their transmission patterns. Anthropotropic variants are mainly associated with water-borne epidemics whereas the enzooitic variants are mainly associated with sporadic cases of HEV (Meng, 2010; Pavio et al., 2010; Geng et al., 2011).

Furthermore, HEV-3 and HEV-4 have a greater range of nucleotide sequence differences at subtype level than HEV-1 and HEV-2. This genetic variability could be associated to their circulation among different animals (Lu et al., 2006). HEV-1 was further divided into 5 subtypes (1a to 1e) and HEV-2 into two subtypes (2a and 2b). HEV-3 is the most heterogenic genotype and was divided into 12 subtypes (3a to 3l), whereas 7 subtypes were described for HEV-4 (4a to 4g) (Lu et al., 2006; de Sabato et al., 2018).

1.4.5 Epidemiology and prevalence.

Attending to HEV prevalences, geographic areas are classified as highly endemic, endemic and non-endemic or sporadic areas (Figure 11).

Highly Endemic Areas

Asia, Africa and America are the continents with the greatest number of endemic areas. India, Bangladesh, Bhutan, Nepal, Pakistan and Sri Lanka are some of these areas in Southern Asia. In central Asia, Kazakhstan, Tajikistan and Uzbekistan are the stand out areas, whereas Burma, Cambodia, Indonesia, Vietnam and Laos are the highly endemic areas in Southeast Asia. On the other hand, Chad, Congo, Gabon, Ivory Coast, Senegal, Liberia, Mali and Nigeria are the highly endemic areas of Africa. Finally, Mexico and Brazil are Central and South American countries with the highest percentages of HEV illness. HEV-1 is the most extended genotype in these countries with the exception of Mexico and some zones in Africa (Senegal, Chad, Nigeria, Congo and Gabon) where HEV-2 is the prevalent genotype (Jacobsen, 2009; Khuroo et al., 2016). The endemicity of HEV infection in these areas is suggested by the occurrence of 7- to 10-year epidemic cycles, similar to the pattern observed for HAV (Kane et al., 1984; Aggarwal and Krawczynski, 2000).

Endemic Areas

In some zones of Africa and South America as well as zones of the Middle East and Southern Asia, HEV causes more than one-fourth of all acute hepatitis or fulminant hepatitis. Turkey, Saudi Arabia,

Yemen, Libya, Bahrain, Oman, Iran, Kuwait and the United Arab Emirates are the Middle East endemic countries, whereas China, Malaysia and Thailand are the Southern Asia endemic countries. Finally, Uruguay in South America also presents HEV as an endemic disease. HEV-1 is the most prevalent genotype in the Middle East zones as well as in Uruguay (like in highly endemic South American areas).

Non-endemic areas/Sporadic Areas

Developed areas as Canada, USA, some European regions, Japan, Russia, Australia and New Zealand, as well as other areas as Peru and Chile, has been developed autochthonous HEV. Except in the north of Japan (where HEV-4 is the usual HEV genotype), HEV-3 is the prevalent genotype in these areas (WHO, 2010; Khuroo et al., 2016). These countries showed rising seropositivity with age, being the highest values of seroprevalence shown in people above 60 years old (Hartl et al., 2016; Khuroo et al., 2016; ECDC, 2017). Only in Europe, the reported HEV cases rose from 514 in 2005 to 5,617 cases in 2015. This increase might be associated with the improvement of HEV diagnosis as well as the awareness of the disease among clinicians (Ijaz et al., 2009, 2013; Pischke et al., 2014; Koot et al., 2015; Adlhoch et al., 2016). Although in these areas HEV is underdiagnosed, this pathogen has been detected more times than other important enteric viruses like HAV (Kuniholm et al., 2009; Lewis et al., 2010; Juhl et al., 2014).

Interestingly, there is also an exception to all of these areas in Egypt, where HEV-1 is the prevalent genotype and occurs at young age with a high seroprevalence among populations. However, HEV-1 subtypes reported from this area are not seen in the surrounded areas (Darwish et al., 1996; Khuroo et al., 2016).

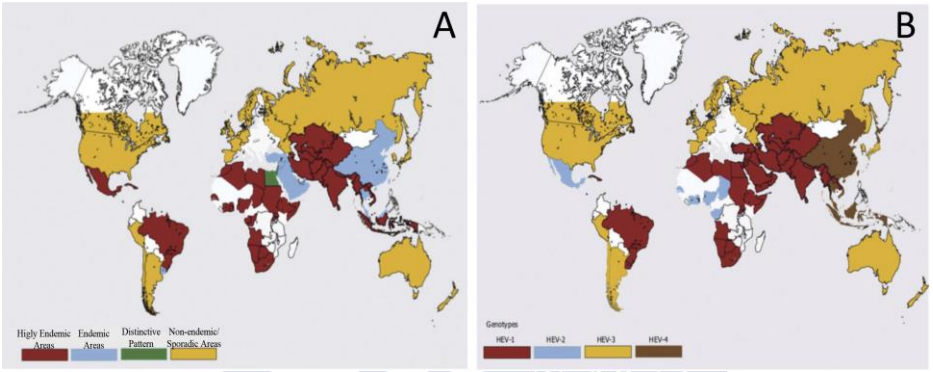


Figure 11. A) Global prevalence of HEV B) Genotype HEV distribution worldwide. (Modified from Khuroo *et al.*, 2016)

1.4.6 Clinical symptoms and relevance.

HEV is considered as an acute self-limiting hepatitis, especially in developed countries. Among symptomatic individuals the incubation period ranges from 22 to 60 days (Melnick, 1957; Balayan et al., 1983). The symptoms described are nausea, dark urine, abdominal pain, vomiting, pruritus, joint pain, rash and diarrhea. Some infected patients present fever and all have hepatomegaly (Khuroo et al.1991). The highest rates of fulminant hepatitis were

reported in pregnant women between 20th and 32nd weeks of gestation. Hemorrhagic diathesis or hemoglobinuria related to renal failure has been the most common causes of death (Devhare et al., 2013). Colestasis with glandlike transformation of bile ducts with preservation of lobular structure, portal inflammation, ballooning degeneration, Kuppfer cell hyperplasia and liver cell necrosis, varying from single cell degeneration to bridging necrosis are also pathomorphological changes observed in HEV patients (Khuroo et al., 1991).

Male to female ratio among symptomatic people ranges from 1:1 to 3:1. The mean age of symptomatic HEV is established in 29 years, with the highest age-specific attack rates in the 20- to 30- year age group (Khuroo et al., 1983; Ricci et al., 2017). HEV virions were detected in stool of human patients approximately 2 weeks before of symptoms beginning or enzyme elevation. HEV viral shedding persists until the liver enzyme elevation returns to normal (Krawczynsky et al., 2000).

Immune response against HEV begins in the early infection stage, IgM antibodies are detectable since the onset of HEV symptoms and persist for 5-6 month after the infection (Purdy et al, 1992; Sharapov et al., 2009). On the other hand, IgG antibodies against HEV have been detected in population 10 years after they were jaundiced during a HEV outbreak and in asymptomatic persons in areas where HEV is endemic (Devhare et al., 2013). Although is not usual, HEV infection could become a chronic disease with prolonged viraemia (more than 3 months) in patients with pre-existing liver disease, organ

transplantation, haematological malignancy or immunocompromised. These HEV affected people present limited symptoms of hepatitis or non-specific symptoms that can derive in liver cirrhosis with fatal consequences (Page et al., 2011; Kamar et al., 2015; Hartl et al., 2016).

There is not specific treatment for HEV infection, although antiviral therapy with ribarin or pegylated interferon-alpha is indicated for the treatment of chronic infections (Izopet 2010; Pol, 2013). A vaccine was developed in 2011 (Hecolin®) based on a recombinant peptide of the HEV-1 genotype, which also shows protection against HEV-4. Due the lack of information, this vaccine has been approved only in China. More studies are needed to enhance the knowledge about possible treatments and future vaccines.

1.4.7 Detection methods

There are many publications about the isolation and propagation of HEV genotypes HEV-1 to HEV-4 in different cell lines (Okamoto, 2011, 2013; Johne et al., 2014). Human liver carcinoma cell lines PLC/PRF/5 and HepG2/C3A, human lung carcinoma cell line A549, as well as cell lines derived from stem cells have been successfully used (Rogee et al., 2013; Talbot et al., 2013; Helsen et al., 2016; Cook et al., 2016) but no cytopathic effect was evident due to HEV replication. Therefore, additional molecular and serological methods are necessary for virus detection.

In immunocompromised people the quantification of HEV RNA is the standard method used for detection of persistent infection (Pischke et al., 2010; Behrendt et al., 2016). ELISA and double-antibody sandwich enzyme immunoassay, targeting ORF2 and ORF3 derived antigens, are the usual serological applied techniques (Zhang et al., 2006; Takahashiet al., 2008; Aggarwal et al., 2016). These detection methods are a valuable alternative for early HEV infection phases, although they have some limitations misdiagnosing HEV in acute infections (Purcell and Emerson, 2008; Pawlotsky, 2014). Combination of serological methods with molecular techniques increases the specificity and sensitivity of testing methods.

As for AiV, PCR techniques are the best options for early diagnosis of HEV in both sporadic and epidemic cases (Gerber et al., 2014; Zhao et al., 2015). There are primers designed to amplify conserved sequences of ORF1, ORF2 and ORF3 that could be detected by conventional RT-PCR or by RT-qPCR (Vollmer et al., 2012; Behrendt et al., 2016; Al-Sadeq et al., 2018). A comparative study between nested-PCR and RT-qPCR was performed (La Rosa et al., 2014), using 24 samples that had been confirmed as HEV positive by anti-HEV IgM and IgG methods, as well as by RT-qPCR. The samples were re-analyzed with different nested-PCR primer sets (targeting the 3 different ORFs), and only 46% of the samples yielded positive results by nested-PCR, indicating that RT-qPCR is more sensitive than conventional PCR techniques (Candido et al., 2012).

Additionally, new detection technologies have been developed for HEV. RT-dPCR has been employed in some studies giving

absolute quantities of HEV RNA. This technique has advantages, since it does not require a standard curve which are often generated with plasmids or transcripts, that result in quantification differences (Nicot et al., 2016; Mykytczuk et al., 2017). The development of these technologies will help to choose the best consensus protocol for detection and accurate diagnosis of HEV in endemic and sporadic areas. Moreover, combination of these techniques will contribute to determine the role of HEV as a causative agent of hepatitis worldwide and its evolution and circulation among human populations.

1.5 Shellfish and enteric viruses

1.5.1 Shellfish as vector

Enteric viruses can be transmitted through the fecal-oral route by direct or indirect contact with contaminated fluids, foods and fomites. The presence of these viruses in the environment is caused by several factors, but mainly due to anthropogenic contamination. Coastal zones are the areas of interaction between the terrestrial and the aquatic environments. Those viruses are discharged into the environment in the feces of the affected individuals. Sewage treatment systems do not guarantee total viral elimination, allowing these pathogens to enter the environment through the discharge of contaminated wastewater. Consequently, high counts of different viral types can be found in wastewater.

After spilling into the environment, viruses can persist for months, both in the water column or associated with sediments. As a

consequence, shellfish obtained from polluted areas may be contaminated with enteric viruses (Nasser, 1994, Callahan et al., 1995, Gantzer et al., 1998).

Bivalve molluscs have been involved for decades as vectors in the transmission of viral diseases (Lindberg-Braman, 1956; Richards, 1987; Lees, 2000). They constitute the differentiated class *Bivalvia* within the Phylum *Mollusca*. The members of this class have bilateral symmetry, are compressed laterally and present a soft body. Molluscs do not present head and are inside a rigid shell formed by two pieces (bivalves). They inhabit the salty and fresh waters where they are dragged to the bottom or are attached to submerged objects in the sand, being sessile or sedentary organisms adaptable to the culture in intensive or semi-intensive conditions (Figure 12.A). Many of these bivalve molluscs are farmed in inshore estuaries where growing waters are also frequently contaminated with human sewage (Iwai et al., 2009; Maalouf et al., 2011).

The mechanism of feeding is by filtration of water from where they take small food particles (Figure 12.B). In the stomach, the mechanical digestion occurs by rotation of the crystalline stiletto on the gastric shield, crunching the food. Two different phases take place: extracellular and intracellular digestion. Extracellular digestion starts as the crystalline style rotates liberating digestive enzymes, and food particles are again selected by size, density and digestibility, being transported to the intestine and eliminated through the anus in the exhaling current or redirected to the digestive diverticula (Gosling,

2003). Studies have shown that most viruses remain accumulated in the stomach and digestive diverticula (Romalde et al., 1994).

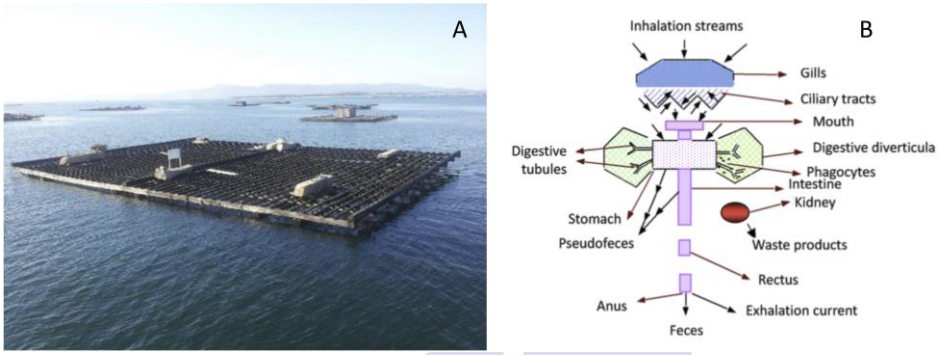


Figure 12. A) Picture of Galician batch used for molluscs growth. B) Scheme representation of mulluscs filtering system

In Europe the main commercialized species are European (*Ostrea edulis*) and Pacific (*Cassostrea gigas*) oysters, common (*Mytilus edulis*) and Mediterranean (*Mytilus galloprovincialis*) mussels, cockle (*Cerastoderma edule*), scallop (*Pectem maximus*) and different species of clams, such as carpet shell clam (*Ruditapes decussatus*), Manila clam (*R. philipinarum*) and the razor clam (*Ensis spp*.).

Whereas the USA legislation established that the levels of the indicators will be evaluated in the shellfish growing waters, the EU Regulations 852/2004, 853/2004, 854/2004; 2073/2005; 1021/2008, 2015/22852019/2229 (Anonymous, 2004 a-c, 2005, 2008, 2015, 2019) establish that the bacterial indicators must be determined in mollusc

meat and in intervalvar water. According with levels of *Escherichia coli* in shellfish flesh, three types of harvesting areas have been established:

-Class A areas: those that present microbiological levels < 230 most probable number (MPN) *E. coli* per 100 g of mollusc. The molluscs harvested in these areas are considered suitable for human consumption, without the need for any further processing.

-Class B areas: those between 230 and 4,600 MPN *E. coli* per 100 g of mollusc. Molluscs from these areas can be marketed after being subjected to a purification process or relocation in an area classified as A.

-Class C areas: those between 4,600 and 46,000 MPN *E. coli* per 100 g of mollusc. Molluscs harvested in these areas could only be traded after a prolonged period of relocation in category A waters or after post-harvest treatment with a heat method.

Molluscan shellfish harvested in other areas that exceed the microbiological levels described above or coming from unclassified areas cannot be marketed for human consumption. However, illegal actions such as the collection from unauthorized areas as well as the elimination of feces of infected persons from ships or boats in coastal areas, or directly on the production zones play a role in the emergence of outbreaks (Desenclos et al., 1991; Dowell et al., 1995; Gerba, 2000; Le Guyader et al., 2010).

Nonetheless, bacteriological indicators are unreliable tools to show viral presence in harvesting areas. In fact, enteric viruses were

detected in molluscs that comply with bacteriological requirements (Chalmers and McMillan, 1995; Richards et al., 2010; Romalde et al., 2002).

NoV and HAV are responsible for most of the shellfish-borne viral outbreaks according to the high number of cases and the severity of the disease (Koopmans and Duizer, 2004). However, emerging viruses, such AiV or HEV, are also a matter of concern since, although less common, they possess the risk of leading to severe disease outcomes (Hollinger and Emerson, 2007; Khamrin et al. 2014; Kamar et al., 2015). Standard methods (ISO/TS 15216-2:2013; ISO 15216-1:2017) for virus detection and quantification in foodstuffs including shellfish are currently available, and the adoption of viral standards into European Union legislation is being under consideration.

1.5.2 Prevention and control methods

Although the most effective approach to prevent the presence of enteric viruses in molluscs would be the control of the entrance of fecal contamination in the production areas, the increasing coastal urbanization and the high investment needed for the implementation of wastewater treatment plants make difficult to avoid this contamination. However, there are different strategies for prevention, control and reduction of microbial contamination in bivalves. These strategies can be grouped attending to the time of implementation into pre-harvesting and post-harvesting strategies.

1.3.3.1 Pre-harvesting strategies

These strategies are focused to obtain an improvement in the quality of the culture waters and the prevention of wastewater discharges. Identification of contamination sources, factors and conditions responsible for the risk of viral contamination in a specific place, as well as the adoption of temporary measures such as the closure of certain production areas, microbial modeling in coastal environments and early warning systems can be useful tools to prevent such contamination.

Two main categories of microbial modelling have been described: statistical models and dynamic models based on processes. Statistical models are based on a linear modeling or logistic regression analysis between environmental parameters and fecal contamination, predicting microbial contamination that results from contributions of wastewater or non-point sources in a given area, according to environmental conditions (Gourmelon et al., 2010). Artificial Neural Network is also an effective alternative statistical model within this category. However, there are limitations in these models due the fact that they do not take into account the type of pollution input, transport, microbial decomposition rate, or spatial and temporal distribution.

Dynamic models are based on three different analyse (Gourmelon et al., 2010):

-A hydrodynamic model that provides pollution mixing and distribution coefficients.

-A dispersion model that integrates the transport and diffusion of bacteria/viruses,

-A biological model of microbial decomposition dependent on environmental conditions (light, temperature and association with sediments).

Finally, the early warning systems are useful tools that record data of certain parameters such as rainfall, salinity variations, sewage network, agricultural activities, climatic condition as well as outbreaks FBO in the local population, dominant mixing and transport processes in points near the production areas (Pommepuy et al., 2005). All these data are used later for the evaluation of risks, notification of events, closing of production areas, calculation of closing time and re-opening date (Le Saux et al, 2006; Gourmelon et al, 2010).

1.3.3.2. Post-harvesting strategies

Although in most viral outbreaks the viral contamination has a pre-harvest origin, there are some methods to eliminate the contamination after the product is collected, especially when culture waters have certain degree of contamination. Depuration, relocation and heat treatment are the three traditional commercial methods applied. In addition, high hydrostatic pressure and irradiation are also relatively new applied strategies.

A) Depuration

Since its introduction more than a century ago (Herdman and Scott, 1896), depuration has significantly reduced the diseases of bacterial etiology transmitted by the bivalves. However, this method is worldwide practiced widely because it allows the marketing of fresh products (Lees et al., 2000), although viral elimination by depuration is much less efficient than bacterial elimination (Polo et al., 2014).

Depuration methods are based on the filter feeding nature of bivalve molluscs. Under correct physiological conditions, the bivalves excrete contaminants in their feces, which settle to the bottom of the tank and are removed at the end of the cycle. The commercial depuration time applied is usually 2-3 days (Lees et al., 2010).

The depuration is carried out in tanks and two different depuration systems are used:

-Open circuit processes: seawater is collected and disposed continuously.

-Closed circuit processes: water is recycled through different sterilization and disinfection systems, as chlorination, UV light, ozonation or iodophors disinfection (Lees, 2000; Richards, 2001).

Both strategies must guarantee specific operating conditions crucial to maintain a normal filtration activity of the molluscs and also its correct performance, avoiding extreme temperatures and blows, good harvesting practices, transport, washing, separation of dead

individuals, as well as contaminated batches, etc (Romalde et al., 2017).

B) Relocation

As in the case of depuration processes, relocation or reinstallation is also based in the feeding filtering nature of molluscan shellfish. It consists on the transfer of molluscs from contaminated areas to other marine contamination-free (class A) areas. Because this is a long-term process, molluscs must be maintained for longer periods (at least 2 months) in the natural environment than in the purification tanks. These facts make reinstallation an adequate process to handle bivalves with a higher degree of contamination (Richards, 1988).

Nonetheless, relocation is less used than depuration. It is because the several difficulties that this strategy presents such as the scarce availability of adequate and pollution-free coastal areas, the vulnerability to poaching or increased associated costs (Lees et al., 2010; Richards et al., 2010; Choi and Kindsley, 2016)

C) Heat treatment

Several thermal treatments have been described for bivalves processing, from simple cooking to pasteurization or sterilization during canning, and constitute the most effective methods for the reduction of viral infection. Furthermore, commercially regulated heat treatment processes have proven their effectiveness for the control of enteric viruses.

However, these methods present some disadvantages. The species and size of shellfish, the level and type of contamination, the conditions and the type of cooking, and the final temperature reached inside the tissues are important factors for viral inactivation (Hewitt and Greening, 2006; Richards et al., 2010). The light cooking, despite its usefulness against surface contamination caused by handling (especially in comparison with raw consumption), is generally inadequate for the elimination of enteric viruses present inside the tissues. A heat treatment at least 90°C for 90 s on the molluscan flesh is considered an adequate treatment to inactivate viral infectivity (EFSA, 2015). Nevertheless, excessive cooking results in organoleptic changes with less commercial acceptance (Lees, 2000; Richards et al., 2010).

D) High hydrostatic pressure

This is a promising method for inactivation of viruses and other pathogens, since does not produces organoleptic changes (Kingsley et al., 2007). Theoretically, the final product maintains the appearance, nutritional quality, taste and texture of the raw product. In addition, this method facilitates the peeling process and extends the shelf life in refrigeration (Murchie et al., 2005). Some studies of NoV inactivation carried out in oysters reported that a pressure of 600 MPa at 6°C for 5 min prevents the risk of infection (Leon et al., 2011). However, these pressures induce a whitish and light cooking appearance that could cause a bad appearance for the consumers.

E) Irradiation

UV and ionizing radiation are also innovative methods with uncertain efficacy for the elimination of viruses in molluscs. UV radiation is effective in the viral reduction on the surface of the product but has no penetration power to inactivate the virus inside tissues (de Roda Husman et al., 2004; Richards et al., 2010). On the other hand, the levels (≥ 3kGy) required to inactivate 90-95% of these viruses with ionizing radiation deteriorates the organoleptic properties of the product, and also imply high business costs (Di Girolamo et al., 1972; Jung et al., 2009; Mallet et al., 1991).

2. JUSTIFICATION AND OBJECTIVES

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2. JUSTIFICATION AND OBJETIVES

Previous studies carried out in our laboratory have demonstrated that NoV, SAV and HAV have an important prevalence in molluscs and clinical samples. The aim of the present doctoral thesis was the acquirement of broader knowledge concerning to Aichi virus and Hepatitis E virus. Both agents are emerging pathogens for which a lack of information existsand limited data is available on the role of the molluscan shellfish on the HEV transmission. The conducted studies presented here were the first AiV and HEV surveys carried out in the main Galician rías (the most important harvesting areas in Europe). The data obtained will increase the quality of the product with an indirect benefit on the aquaculture sector. Furthermore, the present investigations were the first long-term studies of AiV in stool samples in Spain. All this together, brings novel information to the developed analysis.

The present thesis is divided in the next main objectives:

- To evaluate the presence and incidence of AIV and HEV in species of shellfish grown and harvested in Galicia, and suitable for consumption.
- To determine the molecular epidemiology of AiV and their population dynamics by a clinical study in the geographical area of the city of A Coruña.

- To evaluate the genetic diversity of both viruses and to establish phylogenetical relations between the viral strains detected in shellfish and clinical samples.
- To develop an epidemiological study in order to clarify the origin of contamination present in the shellfish harvesting areas.

3. RESULTS

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3. RESULTS

3.1. Study of Enteric virus in Galician Shellfish

3.1.1. Article 2. Low prevalence of Aichi virus in molluscan shellfish samples from Galicia (NW Spain).

Rivadulla E, Varela MF, Romalde JL.
Journal of Applied Microbiology, 122: 516-521 (2017)
Journal Impact Factor 2017: 2.09
Q3, 78/124 in Microbiology.

https://onlinelibrary.wiley.com/doi/full/10.1111/jam.13363

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3.1.2. Article 3. Hepatitis E virus genotype 3 in mussels (*Mytilus galloprovinciallis*), Spain.

Mesquita JR, Oliveira D, Rivadulla E, Abreu-Silva J, Varela MF, Romalde JL, Nascimento MSJ

Food Microbiology, 58: 13-15 (2016)

Journal Impact Factor 2016: 3.7.

Q1, 9/129 in Food Science & Technology (top decile).

https://www.sciencedirect.com/science/article/pii/S0740002015301040?via%3Dihub

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3.1.3. Article 4. Detection of Hepatitis E Virus in Shellfish Harvesting Areas from Galicia (Northwestern Spain).

Rivadulla E, Varela MF, Mesquita JR, Nascimento MS, Romalde, JL

Viruses, 11: 618 (2019)

Journal Impact Factor 2018: 3.811.

Q2, 11/36 in Virology.

https://www.mdpi.com/1999-4915/11/7/618

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viruses

MDPI

Article

Detection of Hepatitis E Virus in Shellfish Harvesting Areas from Galicia (Northwestern Spain)

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Received: 11 June 2019; Accepted: 3 July 2019; Published: 5 July 2019

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Abstract: The hepatitis E virus (HEV) affects almost 20 million individuals annually, causing approximately 3.3 million acute liver injuries, 56,600 deaths, and huge healthcare-associated economic losses. Shellfish produced close to urban and livestock areas can bioaccumulate this virus and transmit it to the human population. The aim of this study was to evaluate the presence of HEV in molluscan shellfish, in order to deepen the knowledge about HEV prevalence in Galicia (northwestern Spain), and to investigate this as a possible route of HEV transmission to humans. A total of 168 shellfish samples was obtained from two different Galician rías (Ría de Ares-Betanzos and Ría de Vigo). The samples were analyzed by reverse transcription-quantitative PCR (RT-qPCR). RT-nested PCR and sequencing were used for further genotyping and phylogenetic analysis of positive samples. HEV was detected in 41 (24.4%) samples, at quantification levels ranging from non-quantifiable ($<10^2$ copies of the RNA genome (RNAc)/g tissue) to 1.1×10^5 RNAc/g tissue. Phylogenetic analysis based on the open reading frame (ORF)2 region showed that all sequenced isolates belonged to genotype 3, and were closely related to strains of sub-genotype e, which is of swine origin. The obtained results demonstrate a significant prevalence of HEV in bivalve molluscs from Galician rías, reinforcing the hypothesis that shellfish may be a potential route for HEV transmission to humans.

Keywords: hepatitis E virus; shellfish; detection; genotyping; food safety

1. Introduction

The hepatitis E virus (HEV) was first documented in a patient with enterically transmitted non-A, non-B hepatitis in 1989 [1]. Nowadays, in addition to hepatitis A, B, C, and D, HEV is one of the major human hepatotropic viruses found around the world. According to the World Health Organization (WHO), HEV affects almost 20 million individuals annually, causing approximately 3.3 million acute liver injuries and 56,600 deaths, with large subsequent healthcare-associated economic losses [2].

HEV belongs to the family Hepeviridae within the genus *Orthopevirus*, which includes five genotypes that infect humans (HEV1, 2, 3, 4, and 7) [3,4]. HEV is a non-enveloped, positive-sense, single-stranded RNA virus, with an icosahedral capsid and a diameter between 27 and 34 nm [5,6]. The RNA genome of HEV is about 7.2 kb and is composed of three open reading frames (ORFs) [7]. ORF1 codes for a nonstructural polyprotein that is essential for viral RNA replication and infectivity. However, it is still debated whether this polyprotein functions as a single multifunctional polyprotein or

undergoes proteolytic cleavage to produce individual active proteins [8]. ORF2 encodes a capsid protein responsible for virion assembly. ORF3 encodes a small protein involved in virion morphogenesis and release [9].

Genotypes 1 and 2 are prevalent in developing countries, including countries in Asia, Africa, and Central America [10,11]. Both genotypes are mainly restricted to humans and are transmitted by the consumption of fecal-contaminated water in areas with poor sanitation [12]. On the other hand, genotypes 3 and 4 have been confirmed as the main causes of zoonotic HEV worldwide; these genotypes represent the main reservoirs found in pigs and wild boars [4,10]. HEV7 has been detected in dromedary camels and in an immunocompromised person who regularly consumed camel meat and milk [3].

Over the last few years, the overall number of reported HEV cases has increased in Europe [13]. Although it is possible for infected humans to infect other people, person-to-person contact is not considered an efficient route of transmission. Other sources of exposure and transmission such as contaminated water, contaminated food (especially of swine origin), or contact with infected animals are more effective routes of transmission [3,10]. The millions of pigs raised in Europe produce huge amounts of manure and it is expected that spillover of such waste may contaminate the environment with HEV [3,14]. Shellfish harvesting areas contain variable amounts of viruses which form a part of the microbial plankton. However, other viruses that are excreted by infected people and animals (even when they are asymptomatic) have also been found in these shellfish harvesting areas, especially when these harvesting areas are located near urban or livestock areas [15,16].

The requirements of shellfish as a protein source has promoted the development of molluscan culture worldwide. Galicia (northwestern Spain) has a particular coastline topography characterized by the presence of fiord-like inlets, called rías, which are especially important as shellfish growing areas. In fact, Galicia is one of the most important regions of mussel production in the world, where they are cultured on floating rafts. Molluscan feeding behavior facilitates the bioaccumulation of pathogens, including viruses [17,18]. In addition, a long viral persistence has been demonstrated in these marine animals [18,19]. These two facts make molluscs important vectors for enteric diseases, especially when consumed raw or lightly cooked [20,21]. The periodic outbreaks of enteric viral diseases transmitted by molluscs have contributed to important economic losses by the seafood industry [22].

In a previous work by our team [23], HEV was detected in Galician mussels harvested from a location with low mussel production that was close to the city of A Coruña, one of the highest population density areas in Galicia (approximately 250,000 inhabitants), suggesting that those viruses were most likely of human origin. The aim of the present study was to perform a systematic surveillance of HEV prevalence in other harvesting areas that have a higher commercial interest and a lower urban influence, in order to confirm the previous results and hypothesis.

2. Materials and Methods

2.1. Shellfish Sampling

A total of 168 samples obtained from two different Galician rías (Ría de Ares-Betanzos and Ría de Vigo) (Figure 1), one in the north of Galician and the other in the south, were analyzed. These samples included wild- and raft-cultured Mediterranean mussels (*Mytilus galloprovincialis*), Manila and carpet-shell clams (*Ruditapes philippinarum* and *R. decussata*, respectively), and cockles (*Cerastoderma edule*) collected from different harvesting areas. Sampling points were classified as B areas (230–4600 MPN *Escherichia coli* per 100 g shellfish) or C (4600-46,000 MPN *Escherichia coli* per 100 g shellfish) according to European legislation [24]. Shellfish samples were collected monthly for 18 months, from January 2011 to June 2012, and were previously analyzed for other enteric viruses [25–27]. The original homogenates prepared from the digestive tissues of 10 mussels or 20 clams or cockles were stored at –80 °C, and were analyzed here for HEV detection.

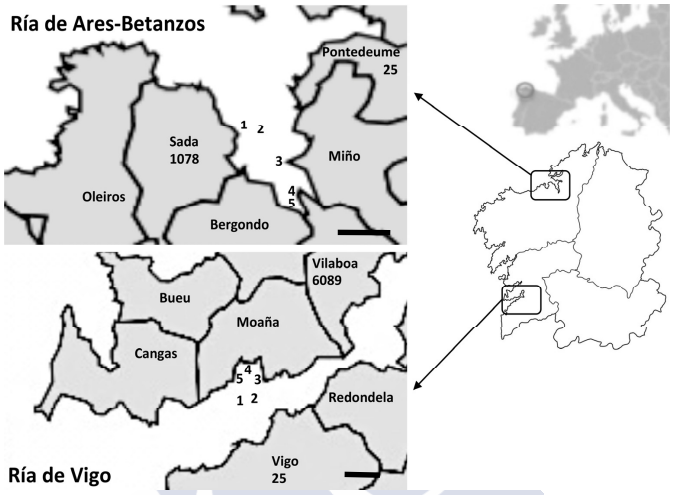

Figure 1. Shellfish sampling points analyzed in Ría de Ares-Betanzos (north) and Ría de Vigo (south). The number of officially registered pigs is indicated under the name of municipalities located near the harvesting areas (data from https://mediorural.xunta.gal/institucional/estatisticas/medio_rural/gando_porcino/). Scale bar, 2 km.

2.2. *Viral Recovery and RNA Extraction*

Viral recovery from shellfish homogenates (2 g) samples was carried out following the ISO 15216-1:2017 specifications, with slight modifications as previously described [28,29]. Briefly, known amounts of Mengovirus clone vMC0 were spiked into each sample homogenate (10 µL, 10^3 plaque forming units [PFU]) as a control for the RNA extraction efficiency [30]. After adding one volume of 0.1% peptone water pH 7.5 (1:1 w/v), homogenates were strongly shaken for one hour at 4 °C, centrifuged at 1000× *g* for 5 min, following which the supernatant was recovered. Viral RNA was extracted in duplicate from each homogenate using a Nucleospin® RNA Virus Kit (Macherey-Nagel; Germany), from a sample volume of 150 µL according to the manufacturer's protocol. The RNA was eluted in RNase-free sterile water and stored at −80 °C.

2.3. *RT-qPCR Assay for HEV Screening and Quantification*

A reverse transcription-quantitative PCR (RT-qPCR) assay targeting the ORF3 region of HEV [31] was applied to undiluted and diluted (1/10) RNA extracts. Negative controls containing no nucleic acid as well as positive controls (viral RNA) were included in each run. RT-qPCR was performed using an iTaq Universal PROBES One-Step Kit (Bio-Rad, Hercules, California, USA) with the primers JVHEVF/JVHEVR and the probe JVHEVP, a TaqMan® probe containing a 5′6-carboxy fluorescein fluorophore and 3´ black hole quencher. The thermal cycling conditions were 42 °C for 5 min and 95 °C for 5 min, followed by 40 cycles of 95 °C for 3 s and 60 °C for 20 s. Extraction and amplification efficiencies were calculated according to the ISO 15216-1:2017 using Mengovirus and appropriate external controls (quantified HEV RNA from a clinical sample) [30,32].

Quantification was also carried out following the principles outlined in the ISO 15216-1:2017 as previously described [28,29]. Briefly, standard curves were constructed using serial dilutions of HEV RNA purified from a clinical sample (kindly donated by Dr. A. Aguilera from the University Hospital

of Santiago de Compostela, Spain), in which the number of genome copies was plotted against the Ct values. Results were expressed as the number of RNA viral genome copies per gram of digestive tissue.

2.4. Broad-Spectrum Nested RT-PCR Assay

Viral RNA from all positive samples detected was subjected to genotyping using a RT nested-PCR protocol designed by Erker et al. [33] with minor modifications. The protocol amplifies the ORF2 region of the HEV genome. Briefly, 5 µL of viral RNA was added to 9.5 µL of the RT mixture from a RevertAid Reverse Transcriptase Kit (Thermo Scientific, USA) with the con-a1 (final concentration 1 µM) primer, in a final volume of 20 µL. The RT reaction was carried out at 42 °C for 60 min.

Next, cDNA samples were amplified by a nested PCR. In brief, for the first-round PCR 20 µL of synthesized cDNA, 0.5 µL of each of the con-a1 and con-s1 primers (final concentration 1 µM) and 4 µL sterile RNAse-free water were added to illustra^{TM} PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Buckinghamshire, UK). The PCR conditions were as follows: a denaturation and activation step at 94 °C for 1 min, followed by 35 cycles of 94 °C for 20 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 10 min.

A 5 µL volume from the first-round PCR product was used as a template for the second-round PCR by mixing it with 0.5 µL of each of the con-a2 and con-s2 primers (final concentration 1 µM) and 19 µL sterile RNAse-free water, and adding it to illustra^{TM} PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Buckinghamshire, UK). The PCR conditions were as follows: a denaturation and activation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 20 s, 55 °C for 30 s, and 72 °C for 40 s, with a final extension at 72 °C for 10 min.

The nested-PCR products were visualized on 2% agarose electrophoresis gel under UV light. Products of the expected length (145 bp) were purified and directly sequenced at STAB Vida Lda. (Portugal). Sequences obtained were processed with the Lasergene 7 software package (DNASTAR Inc., Madison, WI) and were aligned using the MEGA version 7 software package [34]. A phylogenetic tree was built by the maximum-likelihood method with a bootstrap analysis of 1000 replicates. Sequences of HEV reference strains were obtained from GenBank. Sequences of HEV strains detected in the present study are available at GenBank under accessions LR215969 to LR215972.

3. Results and Discussion

Consumption of shellfish has been identified and linked as a risk factor for HEV transmission in Asian countries including China [35], Japan [36,37], Korea [38], Thailand [39], and Vietnam [40]. However, in Europe the route of HEV transmission has not clearly been established. Whilst some investigations point to the absence of HEV in shellfish [41–43], other studies have documented the presence of HEV in wild and commercially available mussels of Italy [44,45], the Netherlands [46], Spain [23,47], and the UK [48,49]. Regardless, few cases of hepatitis E linked to shellfish consumption in Europe have been described so far [40,50].

As mentioned, previous studies carried out by our team detected HEV in Galician mussels from a harvesting area that has a high human influence with prevalence of 14.8% [23]. In the present study, HEV was detected in a higher percentage of the shellfish (24.4%), despite the harvesting areas being located further from urban settlements. Detection in the north estuary Ría de Ares-Betanzos (18 positive samples) was slightly lower than in the southern estuary Ría de Vigo (23 positive samples), yielding prevalence values of 20.0% and 29.4%, respectively. In an attempt to determine the possible origin of such contaminations, and taking into account the negligible urban waste that could arrive to both areas, the presence of porcine livestock around the harvesting areas was analyzed. According to official information (https://mediorural.xunta.gal/institucional/estatisticas/medio_rural/gando_porcino/), 32.8% of Spanish pig farms are located in Galicia, with 28,401 pigs registered. A total of 1103 pigs are bred in pig farms around Ría de Ares-Betanzos, whereas 6114 animals are bred near Ría de Vigo close to the shellfish sampling points (Figure 1). These data could explain not only the higher prevalence in the southern ría, but also indicate a possible swine origin of the HEV detected.

Differences in contamination were also observed among the diverse bivalve species, although without statistical significance (Fisher's exact test; $p > 0.05$). Thus, 20 out of 70 (28.5%) samples of cultured mussels were HEV positive, as well as 13 out of 35 (37.1%) of wild mussel samples. Clams and cockles showed lower prevalences with five out of 31 (16.1%) and three out of 32 (9.3%) positive samples, respectively. Such differences may be due to the different location of the sampling points and the influence of currents or other hydrographic variables, although further studies are needed to confirm these hypotheses. No clear seasonal pattern could be observed, with HEV being detected throughout the studied period (Table 1) with few exceptions, such as the absence of positive samples during February and March for both years in the north estuary. This finding may be explained by the high persistence of the virus in the environment [51] or by a continued supply of virus coming from nearby pig farms.

Table 1. Detection of HEV (◎) along the study period in each harvesting area indicating the present of mixed contaminations with other enteric viruses. Data for norovirus genotype I (■), norovirus genotype II (♦), Aichi virus (✹), Sapovirus (*), and hepatitis A virus (★) were obtained from previous works [25–27].

Zone	Sample [1]	2011												2012					
		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun
Northe	RC mussels (1)				◎ ■							◎ ✹	◎ ✹				◎		
	RC mussels (2)	◎ ♦					◎ ■				◎ ♦						◎*	◎ ♦★	
	W mussels (3)	◎			◎ ■					◎ ■		◎ ♦	◎ ♦.				◎ ♦*		
	Clams (4)								◎ ■	◎ ♦									
	Cockles (5)											◎ ■ ♦*							
South	RC mussels (1)			◎ ■	NT			◎ ■	◎ ■	◎ ■ ♦					◎			◎ ♦	
	RC mussels (2)	◎ ■			NT					◎ ♦			◎ ♦	◎*			◎ ■ ♦		
	W mussels (3)				NT		◎	◎	◎ ■				◎*	◎*					◎ ♦★
	Clams (4)				NT						NT	◎*	◎*	NT		◎	NT	NT	◎ ♦★
	Cockles (5)				NT			◎					◎*	NT			NT	NT	

[1] RC mussels, raft cultured mussels; W mussels, wild mussels; Nt, not tested. The correspondent sampling point is reported in brackets.

The observed prevalence of HEV in the Galician shellfish samples was considerably higher than those reported in other studies in European countries [44–47,49], with the exception of that by Crossan et al. [48], who observed HEV at a prevalence of up to 92% in shellfish from a harvesting area in Scotland located near a slaughterhouse and a meat preparation purification plant that processes pigs. On the other hand, quantification levels in our study ranged from non-quantifiable ($<10^2$ copies of the RNA genome (RNAc)/g tissue) to 1.1×10^5 RNAc/g tissue detected in a cultured mussel sample from the north estuary. Thus, eight samples (19.5%) showed viral levels below the limit of quantification, one sample of cultured mussel from the southern estuary rendered 4.0×10^2 RNAc/g tissue, and the other 31 positive samples (75.6%) showed quantifications ranging from 10^3 to 10^4 RNAc/g tissue, which are in line with the values obtained by Crossan et al. [48].

Phylogenetic analysis based on the ORF2 region showed a high similarity (>96%) among the four HEV sequences obtained from Galician shellfish, regardless of their geographic origin. All sequences corresponded to genotype 3, clustering in the phylogenetic tree with sequences of the sub-genotype

e (Figure 2). Sequences from Galician shellfish were closely related to strains from swine and wild boar of different geographical origins, with similarities in the analyzed amplicons higher than 93.5% [52–54]. Unfortunately, to our knowledge no HEV surveys have yet been performed on porcine livestock in Galicia in order to experimentally confirm the link between shellfish and swine sequences; thus, further studies are needed to uncover this aspect. The same genotype had been previously reported for shellfish from other areas in Galicia [23], as well as in shellfish and water from production areas in Italy [43].

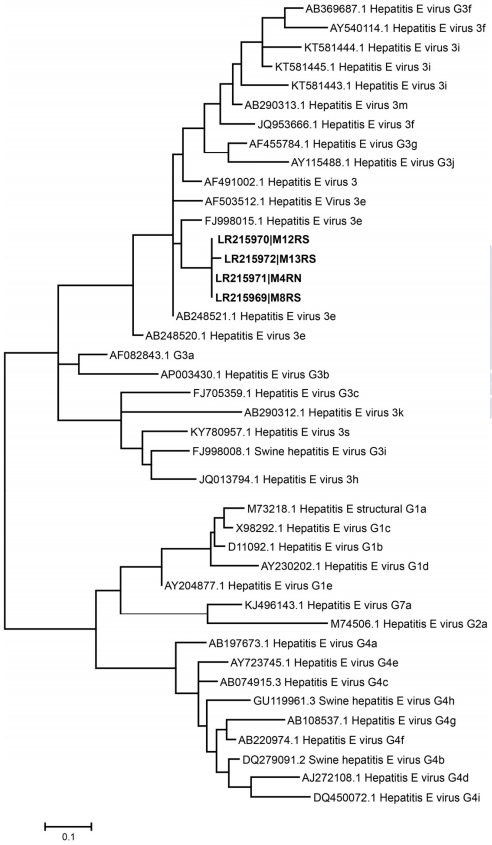

Figure 2. Phylogenetic tree of hepatitis E virus (HEV) samples, based on sequences of the ORF2 region, constructed by maximum likelihood analysis using MEGA 7. GenBank accession numbers of the shellfish (in bold) and reference strains used are detailed in the tree. Scale bar, 0.1 nucleotide substitutions.

The joint analysis of the results of the present study with those of previous work from our laboratory using the same samples [25–27] revealed the presence of mixed contaminations with other enteric viruses. Thus, of 41 HEV positive samples, 34 (83%) showed the presence of more than one virus, and only seven (17%) samples were positive exclusively for HEV. Of these 34 samples, 10 (24%)

were also positive for norovirus (NoV) GI, seven (17%) for NoV GII, seven (17%) for sapovirus (SaV), and two (5%) for Aichi virus (AiV). The other eight (19%) samples showed the co-presence of HEV with two or more viruses (Table 1).

With the exception of the inner most sampling points of the north estuary, which were classified as C areas according to the EU regulations [24], the remaining shellfish samples were obtained from B areas and therefore were considered suitable for human consumption after a depuration process. However, it is important to mention that depuration processes are not completely effective for viral removal [55] and that the viral levels detected in some of the shellfish samples were similar to the theoretical infectious dose reported elsewhere [56]. These facts suggest that shellfish may be a potential route for HEV transmission to humans.

4. Conclusions

The obtained results demonstrated a significant prevalence of HEV in bivalve molluscs from harvesting areas in Galicia and, contrary to previous investigations, an important level of mixed contaminations with other enteric viruses that could strengthen the severity of possible foodborne outbreaks. The evidence obtained in the present work reinforces the idea that pig farms have a strong influence on the appearance of HEV contamination, especially in Ría de Vigo. Although further studies are needed in order to confirm such a hypothesis and to accurately determine the significance of HEV detection in shellfish, the data obtained could be helpful to refine the risk assessment of foodborne transmission of HEV, and will support the establishment of appropriate measures to reduce the risk of shellfish-associated illnesses.

Author Contributions: Conceptualization, J.L.R. and M.S.J.N.; methodology and investigation, E.R., M.F.V., and J.R.M.; formal analysis, E.R. and J.L.R.; writing—original draft preparation, E.R.; writing—review and editing, J.L.R. and J.R.M.; funding acquisition, J.L.R.

Funding: This study was supported in part by grants 10MMA200010PR and 2014-PG110 from the Xunta de Galicia (Spain).

Acknowledgments: The authors gratefully acknowledge Ms. Cristina Álvarez from the INTECMAR (Galicia, Spain) for her help in obtaining the samples included in the analysis. E.R. acknowledges the IACOBUS Program from the European Strategic Group of Territorial Cooperation Galicia/North of Portugal for a research fellowship.

Conflicts of Interest: The authors declare no conflict of interest.

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3.1.4 Article 5. An overview of 20 years of studies on the prevalence of human enteric viruses in shellfish from Galicia, Spain.

Romalde JL, Rivadulla E, Varela MF, Barja JL

Journal of Applied Microbiology, 124: 943-957 (2017)

Journal Impact Factor 2017: 2.09.

Q3, 78/124 in Microbiology.

https://onlinelibrary.wiley.com/doi/full/10.1111/jam.13614

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DE COMPOSTELA

3.2. Presence of Aichi virus in the Galician population

3.2.1 Article 6. Epidemiology of Aichi virus in fecal samples from outpatients with acute gastroenteritis in northwestern Spain.

Rivadulla E, Varela MF, Romalde JL

Journal of Clinical Virology (under revision).

Journal Impact Factor 2018: 3.02. Q2, 17/36 in Virology.

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DE COMPOSTELA

EPIDEMIOLOGY OF AICHI VIRUS IN FECAL SAMPLES FROM OUTPATIENTS WITH ACUTE GASTROENTERITIS IN NORTHWESTERN SPAIN.

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Submitted to: **Journal of Clinical Virology**, June 2019

Revised Ms. JCV-D-19-00034

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Abstract count: 210 words

Manuscript count: 2674 words excluding references

***Abstract**

ABSTRACT

Background: In recent years, Aichi virus (AiV) has been involved in acute viral gastroenteritis outbreaks. However, the common pathogenesis of AiV releases more in subclinical infections underestimating the impact of AiV in human health.

Objectives: The present study describes the presence and genetic diversity of AiV in patients with gastroenteritis in Northwestern Spain.

Study design: A total of 2,667 stool samples, obtained between July 2010 and June 2011, from diarrheic outpatients were studied for detection and molecular characterization of AiV using PCR techniques followed by sequencing and phylogenetic analyses.

Results: The virus was detected in 124 (5.0%) of the samples among all age groups. Coinfections were also detected, from the 124 positive samples, 72 (58.1%) were positive only for AiV, whereas mixed contaminations with *Norovirus* genogroup I or genogroup II, *Sapovirus*, or other enteric pathogens were detected in 52 (41.9%) samples. A total of 70 positive samples could be genotyped, being characterized as genotype A (58.6%) or B (41.4 %). AiV was detected from August to April, being the highest number of AiV positive samples detected during autumn and winter seasons.

Conclusions: This survey remarks the importance of emerging enteric viruses in patients who require medical assistance, and offers more information about the real importance of AiV as gastroenteritis agent.

Keywords: Aichi virus, genotyping, epidemiology, gastroenteritis.

*Highlights (for review)

Highlights

- Prevalence of Aichi virus was determined in outpatients from NW Spain
- The virus was detected in 5.0% of the samples among all age groups
- Monoinfections (58.1%) were more abundant than mixed infections (41.9%)
- Genotypes A and B were detected in all age groups.

1. Background

Viral gastroenteritis constitutes a common human illness, which continues to be a significant cause of morbidity and mortality worldwide [1,2]. *Norovirus* (NoV), *Rotavirus* (RV), *Adenovirus* (AdV) and *Astrovirus* are considered the most important aetiological agents of acute non-bacterial gastroenteritis outbreaks [3,4]. In recent years, human Aichi virus (AiV) has also been involved in acute viral gastroenteritis outbreaks [5].

AiV was first recognized in 1989 from a case of gastroenteritis associated with oyster consumption in Japan [6]. Clinical symptoms include diarrhoea, abdominal pain, nausea, vomiting and fever, but the common pathogenesis of AiV releases more in subclinical infections than in clinically manifest diseases [7,8]. This fact underestimates the real impact of AiV in human health and explains why many studies demonstrated a high prevalence of AiV antibodies in adults (80%-99%), indicating a great exposure to AiV, but a low incidence of AiV in clinical samples from sporadic or epidemic gastroenteritis outbreaks [9].

AiV is a virus with icosahedral morphology that presents a positive-sense single-stranded RNA genome. AiV belongs to the genus *Kobuvirus* within the family *Picornaviridae*, and consists in six recently renamed species: *Aichivirus A*, *Aichivirus B*, *Aichivirus C*, *Aichivirus D*, *Aichivirus E* and *Aichivirus F* [10,11]. *Aichivirus A* is divided in six genetically distinct groups: AiV [7], canine kobuvirus [12], murine kobuvirus [13], Kathmandu sewage kobuvirus [14], roller kobuvirus [15], and feline kobuvirus [16].

Genetically, AiV has been divided in a single serotype and three genotypes: AiV A, common in Europe, Asia, and Africa [17–20]; AiV B, detected in America, Asia, and

Europe [17,21]; and AiV C that was found in a child hospitalized in France that had returned from a trip to Africa [18,22].

Real-time reverse transcription-quantitative PCR (RT-qPCR) assay is a widely used method for AiV identification because it is a rapid and sensitive tool for specific detection and quantitative analysis [23], helpful to determine the circulation of the virus among human populations. Also, conventional RT-PCR coupled with amplicon sequencing has been used for the detection and genotyping of AiV by targeting the viral protein 1 (VP1) [24]. This protein is genetically diverse and useful to establish a timeline for the emergence of AiV variants in different geographic regions.

2. Objectives

Previous studies carried out in our laboratory demonstrated the presence of AiV in Galician molluscs [25]. In this work, a total of 2,667 stool samples from outpatients with acute gastroenteritis in Galicia (Northwestern Spain) were studied for detection and characterization of AiV with the aim to determine its prevalence and predominant genotypes in this region.

3. Study design

3.1. Stool Samples

Stool samples included in this study were obtained from Complejo Hospitalario Universitario de A Coruña, Galicia (NW Spain), which serves more than 550,000 people in an area of 2,750 km^2. A total of 2,667 specimens from outpatients of all ages affected with gastroenteritis were collected during a 1-year period (July 2010–June 2011). For subsequent data analysis, six different age-groups were established: 0–2 years (886 samples), 3–5 years (195 samples), 6–12 years (244 samples), 13–18 years

(71 samples), 19–59 years (653 samples), and >60 years (597 samples). Also, twenty-one samples with unknown age were included.

3.2. Viral stocks

AiV strain A846/88 [7] was kindly provided by Dr. Javier Buesa (University of Valencia, Spain). Mengovirus clone (vMC0) was kindly provided by Dr Albert Bosch (University of Barcelona, Spain).

3.3. Viral recovery and RNA extraction

Viral recovery from original stool samples was carried out as previously described [25]. Briefly, known amounts of Mengovirus clone vMC0 were spiked into each sample homogenate (10 ul, 10^3 PFU) for RNA extraction efficiency control [26]. Supernatants (150 µl) recovered after homogenization in peptone water and centrifugation, were utilized for viral RNA extraction using Nucleospin®RNA Virus Kit (Macherey-Nagel, Düren, Germany). The RNA was eluted in RNase-free sterile water and stored at –80°C.

3.4. RT-qPCR detection and quantification

Viral RNA (5 µl) was tested using Platinum® Quantitative RT-PCR ThermoscriptTM One-step System kit (Invitrogen; France) in a 25 µl total volume, Negative controls containing no nucleic acid as well as positive controls were introduced in each run. The RT-qPCR for AiV was performed on an Mx3005p QPCR System (Stratagene; USA) thermocycler. Extraction and amplification efficiencies were calculated according to the ISO 15216-1:2017 specifications [27] using Mengovirus and appropriate external controls [26,28].

Amplification conditions for AiV were reverse transcription at 45°C for 10 min, denaturation at 95°C for 10 min, followed by 40 cycles of amplification with annealing at 95°C for 15 s and extension at 45°C for 60 s, using the primers described by Kitajima

et al. [29] (Table 1). Quantification was carried out following the principles outlined in the ISO 15216-1:2017 [27] as previously described [25].

3.5. AiV genotyping

Viral RNA of all positive samples was subjected for genotyping using a RT-nested PCR protocol designed by Lodder et al. [24] (Table 1). Amplicons of the expected length were purified and directly sequenced at STABVida Lda. (Portugal). Sequences obtained were processed with Lasergene 7 software package (DNASTAR Inc., Madison, WI) and aligned using MEGA version 6 software package [30]. Phylogenetic tree was built by the maximum-likelihood method (bootstrap of 1,000 replicates). Sequences of AiV reference strains were obtained from GenBank. Sequences of AiV strains detected in the present study are available at GenBank under accession numbers LS479128 to LS479168 and LS481153 to LS481181. AiV sequences obtained from shellfish samples (GenBank accession numbers LS97418 to LS974201) in Galician estuaries [25] were also included in the phylogenetic tree with comparative purposes.

3.6. Statistical analyses

Pearson's chi-squared tests were performed to evaluate differences among AiV prevalences in the different age-groups, as well as to determine correlations among genotypes and age-groups. Analyses were carried out using IBM® SPSS® Statistics 20 software (IBM Corp., USA).

4. Results

4.1. AiV prevalence

All stool samples showed acceptable RNA extraction (>5%) and RT-qPCR (>25 %) efficiencies. AiV were detected in 124 (4.7%) of the total samples, being present in patients of all age. The highest prevalence was observed in children between 3-5 years,

(5.6%), followed by patients between 19-59 years (4.9%). In infants under 2 years, AiV were observed in 4.9% of the patients. Other age groups showed lower prevalences, 4.2% in teenagers between 13-18 years old, 4.1% in children between 6-12 years old, and 3.7% in people older than 60 years (Figure 1). No significant statistical differences ($p>0.05$) were detected for the AiV prevalences among the several age-groups.

4.2. Mono and mixed infections

The AiV detection was comparatively analyzed with results previously obtained in our laboratory in the same stool samples [31,32], in order to detect coinfections of AiV with other enteric pathogens.

Just over half of the positive samples (58.1%) appeared as AiV monoinfections (Figure 2). The 0-2 years, 19-59 years and >60 years were the age groups where most of these monoinfections were detected (Supplementary Table 1). Only in the age group 13-18 years all the positive samples constituted monoinfections of AiV, but it is also noteworthy that it was the age-group with a lowest number of samples analyzed (Supplementary Table 1).

Mixed infections comprised a variety of enteric pathogens, including viruses, bacteria and parasites (Figure 2; Supplementary Table 1). Among these, the more abundant were coinfections with other viruses. Coinfections of AiV and *Sapovirus* (SaV) were detected in 11 samples, followed by coinfections of AiV with NoV genogroup I (GI) (7 samples) or NoV genogroup II (GII) (7 samples). Three viral types were detected in 10 samples (5 with AiV, NoV GI and SaV, and 5 with AiV, NoV GII and SaV). A random distribution of these mixed infections among the different age groups was observed, although in general were more abundant in groups 0-2 years, 3-5 years and 19-59 years (Supplementary Table 1). No coinfections by AiV, NoV GI and GII were detected.

Co-infections only with bacterial pathogens were detected in 9 of the AiV positive

125 samples (Figure 2; Supplementary Table 1). Most cases of these mixed infections were
126 with *Campylobacter* spp. (4 samples), followed by *Salmonella* spp. (3 samples) and
127 *Aeromonas* spp. (2 samples), being generally more abundant in patients under 2 years.
128 One sample in the group 0-2 years rendered positive for AiV and the parasite
129 *Cryptosporidium* (Figure 2; Supplementary Table 1).
130 In a total of 7 samples, the presence of three or four bacterial and viral agents was
131 detected (Figure 2; Supplementary Table 1). Thus, triple infections of AiV, SaV and
132 *Campylobacter*, AiV, SaV and *Yersinia enterocolitica* or AiV, NoV GII and *Yersinia*
133 *enterocolitica* were observed in patients of 0-2 years. Multiple infections by AiV, NoV
134 GI, SaV and *Campylobacter* were detected in two samples, one in the group 0-2 years
135 and the other in the group 3-5 years. Other polyinfections included AiV, NoV GI, SaV
136 and *Bacillus cereus*, detected in a sample from the group 19-59 years, and AiV, NoV
137 GII, SaV and *Campylobacter*, detected in a sample from the group 6-12 years (Figure 2;
138 Supplementary Table 1).
139 *4.3. AiV quantification*
140 Quantification levels for AiV in feces ranged from 7.14 x 10^2 GC/g, detected in a
141 clinical sample from the age group of >60 years old, to 6.38 x 10^8 GC/g, detected in a
142 stool sample from a child in the age group of 0-2 years with a mixed infection of AiV,
143 NoV GII and SaV. The mean values of AiV were also estimated by age group, ranging
144 from 9.81 x 10^3 GC/g obtained for the age group of ≥60, to 4.12 x 10^4GC/g obtained for
145 the age group of 0-2 years (Table 2).
146 *4.4. Epidemiology*
147 Seasonally, AiV was detected from August 2010 to April 2011, although the highest
148 number of positive samples was observed during autumn and winter seasons (Figure 3).
149 October was the month with more positive samples followed by January, September

and February, 85.4% of the positive samples being detected in these 4 months.

Seventy out of 124 positive samples could be genotyped. Forty-one (58.6%) samples belonged to genotype A and 29 (41.4 %) samples to genotype B (Table 3; Figure 4). Genotype C was not observed in this study. Most of genotype A samples were related to strain A846/88, originally isolated from an oyster-associated gastroenteritis outbreak in Japan (Figure 4). Genotype A and B sequences were present in samples from all groups. The genotype A was especially abundant in infants under 2 years (22.9%) while genotype B was more prevalent in adults between 19-59 years (20%) (Table 3), being such correlations statistically significant ($p<0.05$). Finally, the 4 molluscan samples included were classified as genotype B and two of them were phylogenetically related to clinical samples with similarities higher than 97.5% (Figure 4).

5. Discussion

Aichi virus has emerged in the last years as a gastroenteritis agent of considerable importance in different parts of the world, as evidenced by different seroprevalence studies [17,18,33,34]. Isolation of the virus from patients with gastroenteritis was reported in Japan [35], Southeast Asia [19,21], Germany and Brazil [17]. In addition, AiV RNA was detected in stool samples from some other European countries, including France, Hungary or Finland [18,36,37]. The present study constitutes the first survey on prevalence of AiV in Spain using molecular methods for direct detection of viral RNA.

AiV prevalence (≈ 5%) observed in the present study, although at levels sligthly higher, is in agreement with the low incidence reported in other surveys from other geographic regions [19,22,38]. As previously described, this low prevalence contrasts with the results obtained from seroepidemiological studies [17,35,39], which indicate that practically all the population with age >50 show antibodies to AiV. Specifically in

Spain, a high seroprevalence (>85%) was observed for people over 20 years old, suggesting a general exposition to this human pathogen in our country [33] probably through subclinical infections.

Our results show no great differences among the different age groups, all of them with prevalences around 4-5%. These results are in contrast with those reported from Germany [17], where most of infections seem to occur among children younger than 6 years. Most of studies on AiV prevalence have been focussed on children cohorts [18,20,35,36]. Prevalences obtained in the present study for the age groups 0-2 years (4.85%) and 3-5 years (5.64%) are higher than those observed in France [18], Hungary [37] or Finland [36], and at comparable levels to those observed in Tunisia [20].

It has been suggested that the presence of AiV in samples from gastroenteritis outbreaks could be considered as an indicator of mixed infections [18]. However, the results obtained here, rendering a high percentage of monoinfections (58.1%), seem to support the hypothesis of Sdiri-Loulizi et al. [34] who suggested the role of AiV as a real pathogenic agent virulent enough to cause the need of medical care and/or hospitalization.

Certain geographical distribution of the AiV genotypes can be deduced from the literature. Thus, genotype A is predominant in Japan and has also been detected in Germany and France [17–19]. Genotype B is predominant in Bangladesh, and has been observed in Brazil, Pakistan, Malaysia, and Nigeria [17,19,21,40]. Similar prevalence of these two genotypes was reported in Finland [36], although the number of positive samples was too low to obtain firm conclusions. On the other hand, genotype C was detected once in France from one patient returning from a trip to Africa [18].

In the present study, genotypes A and B were detected, showing genotype A sligthly higher prevalence. It is interesting to point out that genotype A was more prevalent in

infants of 0-2 years, whereas genotype B was more abundant in the age group 19-59 years. On the other hand, the AiV detected in molluscan samples in the same geographic area were characterized as genotype B, showing high similarity with some clinical samples. Such results may indicate that contaminated shellfish may constitute a via of transmission of AiV within human population in this area, and that feed habits may be responsible in part of the genotype-drift from genotype A to B with the age. Taking togeteher all this data, the replacement of viral types over the age groups could be explained by variations in transmission of AiV by direct contact, food, or travelling [41], although further studies are needed to gain more knowledge on the genotype distribution worldwide.

Although AiV was present along the year, different monthly prevalences were observed being the majority of positive samples concentrated in autumn and winter months. Factors like climatological and oceanographic conditions, international travelling, or seasonality of mollusc consumption could influence to these seasonal peaks [42].

In summary, recent molecular methods for screening and characterization of gastroenteritis pathogens revealed novel enteric virus as the cause of diarrhoeal illness and outbreaks. The present study confirms the presence of AiV in gastroenteritis patients, offering more information about the importance of this virus on human health in our region. However, it is important to point out that taking together these results and those from previous works in our group, the aetiology could only be established for aproximately 50% of all the gastroenteritis cases studied. This fact suggests that other enteric pathogens may be present and further studies are needed in order to identify them and to determine their real public health importance.

225 **Conflict of interest**

226 The authors declare no conflict of interest.

227

228 **Funding**

229 This study was supported in part by was supported in part by Consellería nde
230 Educación, Universidade e Formación Profesional, Xunta de Galicia, Spain (Grant
231 2014-PG110).

232

233 **Acknowledgments**

234 The authors deeply thank Dr. Germán Bou from the complejo Hospital Universitario de
235 A Coruña (Galicia, Spain) for the original samples included in the analysis, and Mr.
236 Alberto Lema his help with the statistical analysis.

237

238 **Author's contributions**

239 JLR designed the work. ER and MFV performed the experiments. ER and JLR analyzed
240 the data. ER and JLR wrote the paper. All authors revised the manuscript, read and
241 approved the final draft. JLR supervised the study.

242

243 **Ethical approval**

244 This study was carried out in accordance with the Declaration of Helsinki as revised in
245 2000. This non-interventional study included no additional procedures. Anonymized
246 biological material was obtained only for standard viral diagnosis. The Spanish
247 Biomedical Research law (14/2007; article 3i) does not require written informed
248 consent for such a protocol.

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410
411
412 **Figure legends**
413
414

415 **Figure 1.** Human AiV prevalence within the different age-groups.

416

417 **Figure 2.** Total number of AiV cases in coinfection with other viral and bacteriological
418 agents. Data for comparison obtained from Manso and Romalde [31] and Varela et al.
419 [32].

420
421 **Figure 3.** Distribution of the AiV prevalence along the period of study.

422
423 **Figure 4.** Phylogenetic tree of AiV samples based on VP1 region sequences by neighbor-
424 joining analysis using MEGA 6. Galician samples are shown in bold type. Asterisks
425 indicate sequences obtained from shellfish samples. Bootstrap values (greater than 50%)
426 are shown at each node as percentages of 1,000 replicates. GenBank accession numbers
427 are detailed in the tree. Bar, nucleotide substitutions per site.

428

Tables 1-3

Table 1. Primers and probe employed in the study for AiV detection and sequencing

Primers and Probes	Sequence (5´-3´)[a]	Nucleotide location	Reference
RT-qPCR Primers			[29]
AiV-AB-F	GTCTCCACHGACACYAAYTGGAC	1882–1904[b]	
AiV-AB-R	GTTGTACATRGCAGCCCAGG	1970–1989[b]	
RT-qPCR Probe			[29]
AiV-AB-TP	FAM-TTYTCCTTYGTGCGTGC-MGB	1939–1955[b]	
RT-Nested PCR primers			[24]
AiV-VP3-F1	CACACCGCCCCTGCGTCRGCCCTCGT	2912–2937[c]	
AiV-VP1-F2	CTCGATGCRCCMCAAGACACCGG	3023–3045[c]	
AiV-VP1-F3	GTGCTTCACRTACATCGCYGCGG	3289–3311[c]	
AiV-VP1-R2	CCTGACCAGTCCTCCCAWCCGAAGTA	3552–3527[c]	
AiV-VP1-R1	GAGAGCTGGAAGTCRAAGGG	3651–3632[c]	

[a]Mixed bases in degenerate primers and probe are as follows: H represents A, C, or T; R represents A ; Y represents C or T; M indicates A or C and W indicates A or T.
[b]Position of the AiV primers is of the 5´base relative to AiV reference strain no. AB040749.
[c]Position of the AiV primers is of the 5´base relative to AiV reference strain no. AB010145.

Table 2. Quantification of AiV levels (log genome copies/g feces) by age group.

Age group (yr.)	Mean	SD	Range
0-2	4.61	8.00	3.00-8.80
3-5	4.56	6.70	3.31-7.22
6-12	4.46	7.71	3.54-8.21
13-18	4.15	4.21	3.80-4.57
19-59	4.36	7.58	3.34-8.34
≥60	3.99	6.01	2.85-6.67

Table 3. Genotype of sequenced samples by age group

Age group	Genotype A		Genotype B	
	No. of positives	%	No. of positives	%
0-2 yr	16	22.9	6	8.6
3-5 yr	3	4.3	4	5.7
6-12 yr	4	5.7	1	1.4
13-18 yr	2	2.9	0	0.0
19-59 yr	9	12.9	14	20.0
> 60 yr	7	10.0	4	13.8
Total	41	58.6	29	41.4

Figure 1
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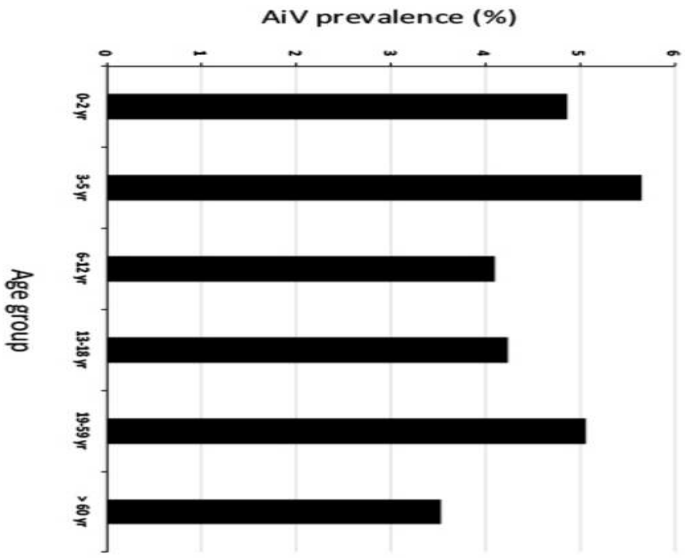

Figure 2
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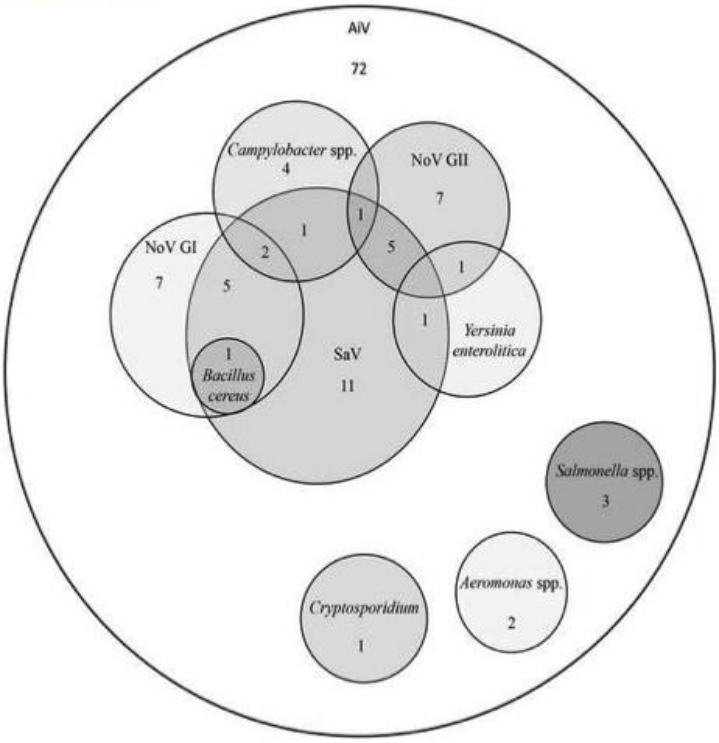

Figure 3
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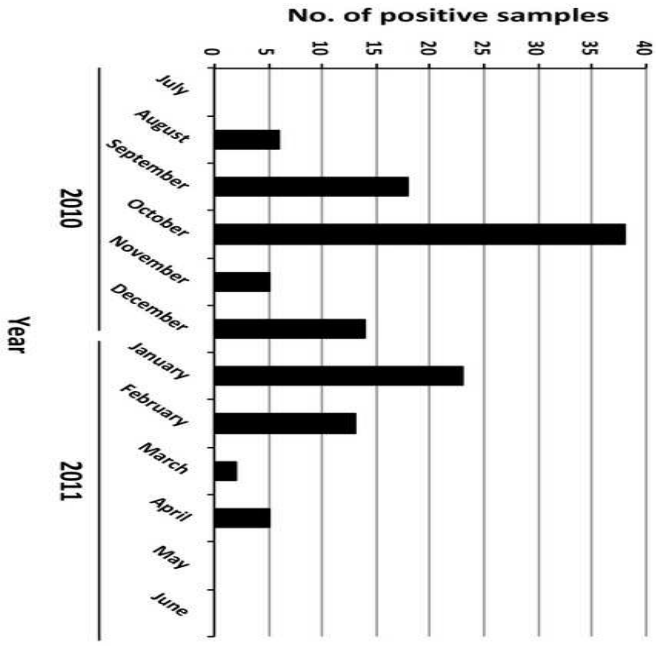

Figure 4
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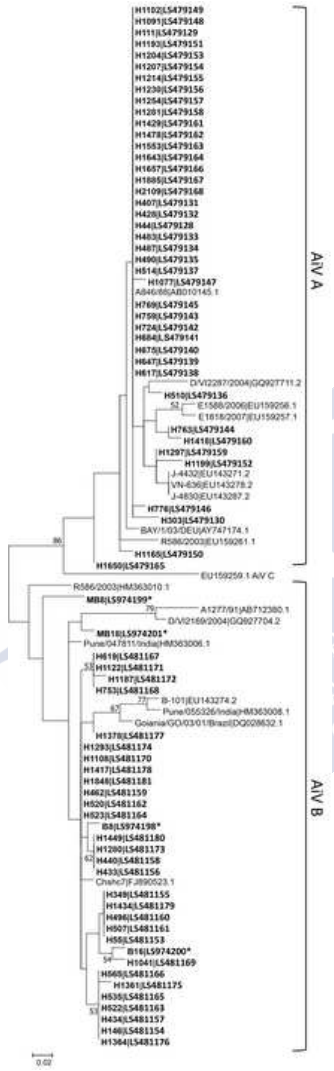

Supplementary Data

EPIDEMIOLOGY OF AICHI VIRUS IN FECAL SAMPLES FROM OUTPATIENTS WITH ACUTE GASTROENTERITIS IN NORTHWESTHERN SPAIN.

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Supplementary Material

Supplementary Table 1. Distribution of AiV in mono- and mixed infections in the different age groups.

Agent	Age group							Total
	0-2	3-5	6-12	13-18	19-59	>60	Unclassified	
AiV	17	5	7	3	21	17	2	72
AiV + SaV	2	3	1	0	4	1	0	11
AiV + NoV GI	2	0	0	0	3	1	1	7
AiV + NoV GII	5	2	0	0	0	0	0	7
AiV + NoV GI + SaV	3	0	1	0	0	1	0	5
AiV + NoV GII + SaV	4	0	0	0	1	0	0	5
AiV + *Campylobacter*	3	0	0	0	1	0	0	4
AiV + *Salmonella*	1	0	0	0	2	0	0	3
AiV + *Aeromonas*	1	0	0	0	0	1	0	2
AiV +NoV GI + SaV + *Campylobacter*	1	1	0	0	0	0	0	2
AiV + *Cryptosporidium*	1	0	0	0	0	0	0	1
AiV + SaV + *Campylobacter*	1	0	0	0	0	0	0	1
AiV + SaV + *Yersinia enterocolitica*	1	0	0	0	0	0	0	1
AiV + NoV GII + *Yersinia enterocolitica*	1	0	0	0	0	0	0	1
AiV + NoV GII + SaV + *Campylobacter*	0	0	1	0	0	0	0	1
AiV +NoV GI + SaV + *Bacillus cereus*	0	0	0	0	1	0	0	1
TOTAL	43	11	10	3	33	21	3	124

***Credit Author Statement**

Author's contributions

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript.

JLR designed the work. ER and MFV performed the experiments. ER and JLR analyzed the data. ER and JLR wrote the paper. All authors revised the manuscript, read and approved the final draft. JLR supervised the study.

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4. DISCUSSION

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4. DISCUSSION

On the past years, the inefficiency of current employed methods for viral control due to the absence of standardized methodologies, has become clear. However, with the implementation of ISO guidelines (ISO/TS 15216-2:2013; ISO 15216-1:2017) these problems have been solved in part. Viral surveys of molluscs based on techniques such as RT-qPCR reflect the risk that this type of food can present for consumers. These methodologies also reveal the circulation of viruses between population and shellfish harvesting areas. Additionally, they provide useful information for the establishment of future virological legislation and to analyze the impact of these pathogens on the aquaculture sector (Polo et al., 2015).

Several studies carried out in our laboratory have demonstrated that NoV, HAV or even SaV are important aetiological agents of AG present in Galician shellfish samples (Manso et al., 2010; Manso et al., 2014; Polo et al., 2015; Varela et al.,2016a/2016b). This thesis adds novel information about epidemiology and aetiology of AiV and HEV.

All the studies carried out in the present thesis follow the cited ISO guidelines with minor modifications. External controls for extraction and amplification efficiency evaluation were added. Samples rendered valid extraction (>5%) and amplification (25%) efficiencies and not significant differences among different molluscan

species, seasons or geographic areas were observed, conferring consistence to the reported results.

Due to its importance on the aquaculture sector the most studied species were cultured and wild mussel (*M. galloprovincialis*), but other bivalve species were also analyzed. The present work constitutes the first systematic study on the presence of AiV and HEV in Galician molluscs.

The results clearly show that HEV is more prevalent than AiV. Overall, HEV was detected in 53 (21.2%) out of 249 of the analyzed molluscan samples. On the other hand, the prevalence of AiV reported is lower and the virus was detected in 15 (6.0%) samples distributed among the three rías. This predominance of HEV is also observed in the quantification levels. While HEV ranged from non-quantifiable to 1.1 x 10^5 RNAc/g tissue, AiV ranged from non-quantifiable to 6.1 x 10^3 RNAc/g tissue.

In the north estuaries HEV was reported in 18 (20%) out of 90 samples from Ría Ares-Betanzos and 12 (14.8%) out of 81 samples from Ría do Burgo, whereas AiV was detected in 10 (11.1%) and 3 (3.7%) samples respectively. On the other hand, in the south HEV was detected in 23 (29.4%) out of 78 samples from Ría de Vigo and AiV in only 2 (2.5%) samples.

The prevalence of HEV over AiV in some of the harvesting areas, namely Ría de Ares-Betanzos and Ría de Vigo, could be explained considering the pig farms located near the estuaries. The thousands of pigs raised near the *rías* produce hundreds of tons of contaminated manure. This idea was also reinforced by the

phylogenetic analysis, based on the ORF2 region, that showed a high similarity among the obtained sequences. All sequences, distributed along the three rías, corresponded to genotype 3 subgenotype e, being closely related to strains of swine and wild boar origin. However, as mentioned before, no HEV surveys were performed yet on the porcine livestock in Galicia in order to confirm this relation and further studies will be needed in order to confirm this route of contamination.

Comparing the obtained results with those of previous works carried out in our laboratory, the majority of positives samples, for both HEV and AiV, were also contaminated with other enteric viruses (Manso et al.,2013; Polo et al., 2015; Varela et al., 2016).

The other main objective of this thesis was the study of the prevalence of AiV among Galician population. For that purpose, the AiV prevalence was analyzed in patients that were suffering acute gastroenteritis. Although a previous survey indicated a high seroprevalence among Spanish population (Echeverarria et al., 2014), there was not long-term studies of AiV in stool samples in Spain.

The obtained results were in agreement with the observed prevalence in molluscs. AiV was detected in only 124 (5%) out of 2,667 clinical samples. However, the amount of monoinfections observed (58.1%) support the proposed hypothesis of AiV as a real pathogenic agent, virulent enough to require medical care and/or hospitalization (Sdiri-Loulizi *et al*., 2010). Nonetheless, it is important to point out that, including the present and previous works from our research group, the aetiology could only be established for aproximately 50% of all the gastroenteritis cases studied. This fact

suggests that other enteric pathogens may be present and further studies are needed in order to identify them and to determine their real public health importance.

Furthermore, phylogenetic analyses based on VP1 region of AiV genome showed high similarities between shellfish and some clinical samples. Whereas genotype A was prevalent in infants of 0-2 years, genotype B was more abundant in patients of 19-59 years being also the only genotype reported from molluscs. The results suggest that this kind of food could be involved on the drift from genotype A to genotype B with the age, due the variations in alimentary habits produced throughout life.

Although no seasonal trend was clearly concluded for AiV, the pathogen was detected mostly in cold months with some summer peaks. Factors like climatological and oceanographic conditions, increases of travel or seasonality of mollusc consumption could influence to this summer peaks (Romalde et al., 2017).

However, it is fair to indicate that, although the results could suggest the cited relation between shellfish consumption and disease development, the epidemiological information (i.e. food consumption, personal relation, family data, etc) needed to establish such relationship in a reliable way is unknown. Furthermore, the limitations of PCR methodologies make even more difficult this task, as these methods only detect presence of viral nucleic acid and not infective particles. Therefore, the real risk of infection and the role of bivalve mollusc for as vectors for both studied pathogens remain unclear.

Further investigations will be needed for a better understanding of the significance of AiV and HEV detected in shellfish.

In summary, recent molecular methods for screening and characterization of enteric pathogens revealed the importance of the emerging enteric viruses as the cause of outbreaks of diarrheal disease. Our results confirm the presence of AiV and HEV in Galician harvesting areas, the most important bivalve producer in Europe and one of the most important producers in the world. These results reinforce the necessity of adequate viral controls for shellfish and their harvesting areas not only based on bacterial indicators.

The appearance of mixed viral contaminations is a fact that should be taken into account in the future. In this sense, we hope that the discoveries achieved could contribute to fill a certain knowledge gap existing in this field of study.

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5. CONCLUSIONS

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5. CONCLUSIONS

From the results obtained in this work it can be concluded that:

1. It was demonstrated a significant prevalence of hepatitis E virus in bivalve molluscs from harvesting areas in Galicia and an important level of mixed contaminations with other enteric viruses, that could strengthen the severity of possible foodborne outbreaks.
2. A strong influence of pig farms on the appearance of Hepatitis E virus contamination in shellfish harvesting waters was evidenced.
3. The presence of Aichi virus was, for the first time, confirmed in Galicia (Spain), both in shellfish and in patients suffering from acute gastroenteritis.
4. The high proportion of Aichi virus monoinfections detected in clinical samples support the hypothesis of this virus as a real pathogenic agent, virulent enough to require medical care.
5. A genotype drift from genotype A to B was observed, being genotype A more prevalent in infants of 0-2 years and genotype B more abundant in the age group of 19-59 years. The changes on the feed habits could be responsible in part of the genotype drift.
6. The relation among clinical and molluscan samples suggest that this bivalve shellfish may constitute a route of transmission for these viruses.

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6. RESUMEN EXTENDIDO

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6. RESUMEN EXTENDIDO

La ingesta de comida contaminada da lugar a enfermedades alimentarias, siendo los agentes de tipo biológico (bacterias, virus priones, protozoos y otros tipos de parásitos) los principales causantes de este tipo de infecciones. No obstante, el papel que juegan estos agentes biológicos en las enfermedades de origen alimentario es difícil de determinar. De hecho, esta ruta de transmisión era desconocida hasta finales del siglo XIX.

Aunque principios de ese siglo algunas enfermedades infecciosas como la sífilis o la viruela se consideraban contagiosas, hubo un amplio debate sobre el origen de otras como el cólera. El modelo predominante para explicar la transmisión de esta última fue la teoría "miasmática". Según esa teoría, las miasmas eran un conjunto de emanaciones fétidas y toxicas para las personas liberadas al aire y originadas en suelos y aguas impuras.

El punto de inflexión se produjo durante un brote de cólera ocurrido en Londres entre los años 1853-1854. Fue durante ese tiempo cuando John Snow localizó las casas de los individuos enfermos y las fuentes públicas de agua, concluyendo que la mayor cantidad de muertes se producía cerca de un surtidor público de agua en Broad Street. Esta correlación entre la incidencia del cólera y la fuente de agua pública confirmó su teoría sobre la transmisión

del cólera por agua contaminada, en lugar de la creencia popular de que la enfermedad se transmitía por el aire. Finalmente, la teoría de la transmisión por el agua fue aceptada en 1866. Desde entonces, los métodos para la detección de patógenos transmitidos por los alimentos han mejorado y las rutas de transmisión son más claras.

Es necesario que se den una serie de situaciones para poder establecer el origen de los brotes de gastroenteritis. En primer lugar, la persona o grupo de personas afectadas deben requerir atención médica. Una vez recibida, el personal hospitalario debe solicitar una muestra clínica para la que el paciente, o pacientes, deben dar su consentimiento. Después de eso, los investigadores deben identificar el agente causal y, finalmente, los resultados del diagnóstico deben informarse para ser analizados.

Si se omite alguno de estos pasos, el patógeno causante de la enfermedad no se diagnosticará ni se notificará. Además, hoy en día hay muchos alimentos elaborados con múltiples ingredientes que, relacionados con la escasa memoria de los pacientes acerca de los productos consumidos, junto con los largos períodos de incubación de algunos de estos patógenos, hacen que la identificación sea complicada.

Cada año, se estima que los patógenos transmitidos por alimentos afectan aproximadamente a 48 millones de personas, lo que da como resultado 127.839 hospitalizaciones y 303 muertes. Solo en la Unión Europea y los países circundantes, alrededor de

23 millones de personas se infectan anualmente. De hecho, este número ha ido en aumento desde principios del siglo XXI.

Las implicaciones económicas que acarrean las enfermedades de transmisión alimentaria también son considerables. Para hacerse una idea, solo en Estados Unidos se estima que causan una pérdida económica que oscila entre 55,5 y 93,2 miles de millones de dólares al año.

El rápido crecimiento de la población mundial sumado al comercio globalizado entre países son algunos de los principales factores que contribuyen hoy en día a la propagación de las enfermedades de transmisión alimentaria. Los datos epidemiológicos siguen siendo escasos en muchos países en vías de desarrollo lo que, sumado al carácter autolimitante de muchas de estas infecciones, hace que la mayoría de los brotes pasen totalmente desapercibidos por los servicios sanitarios y las autoridades competentes.

Además, aunque las enfermedades de tipo gastrointestinal suelen estar asociadas a virus entéricos, es común que estos virus se vean enmascarados por otros patógenos que causan un cuadro clínico similar. De entre todos los tipos de agentes microbianos causantes de este tipo de enfermedades, Rotavirus (RV), Norovirus (NoV), Hepatitis A (HAV), Sapovirus (SaV) *Campylobacter spp*. y *Salmonella* son los más frecuentes. Sin embargo, en los últimos años se han descrito otros virus emergentes transmitidos por los

alimentos como el virus de la hepatitis E (HEV) y Aichi virus (AiV) capaces de causar brotes de enfermedad.

En lo que se refiere a AiV, tal y como se recoge en el artículo de revisión incluido en la introducción de esta memoria (*Article 1. A Comprehensive Review on Human Aichi Virus*), es un virus entérico emergente que se incluye en el género *Kobuvirus* de la familia *Picornaviridae*. Los viriones muestran una morfología icosaédrica que presentan un RNA monocatenario de sentido positivo (8.280 nucleótidos) y una cadena poly (A). Fue detectado por primera vez después de un brote de gastroenteritis aguda asociado al consumo de ostras en la Prefectura de Aichi (Japón) en 1989. Además, diversos estudios llevados a cabo en Japón, Alemania, Francia y Túnez demostraron una gran prevalencia de anticuerpos de AiV en adultos (entre el 80 y el 99%) lo que es indicador de una gran exposición a este virus. También se han observado grandes concentraciones en aguas residuales y de río, lo que sugiere que se mantienen en humanos de forma asintomática o causando gastroenteritis en pacientes no hospitalizados.

La diarrea, el dolor abdominal, las náuseas, los vómitos y la fiebre son los síntomas clínicos usuales provocados por los virus entéricos, pero la patogénesis de AiV reside más en infecciones subclínicas que en enfermedades clínicamente manifiestas, lo que puede llevar a una infravaloración de su verdadero riesgo para la salud publica

De hecho, el verdadero papel de AiV como patógeno gastrointestinal sigue siendo poco claro. Algunos estudios sostienen la idea de que solo puede causar un brote cuando otros patógenos entéricos están presentes, debido a su frecuente co-detección con otros agentes gastrointestinales. Sin embargo, otros estudios muestran que la coinfección de AiV con otros virus entéricos se produce en un porcentaje bajo y, además, al no estar presente en individuos sanos, reforzaría la idea de que AiV es un patógeno gastrointestinal importante.

Cuando se produce, la infección por AiV es generalmente leve y dura de dos a tres días. La replicación de AiV produce la destrucción de la capa de enterocitos que cubre el tercio superior de las vellosidades intestinales interrumpiendo la reabsorción de agua. Como respuesta, las vellosidades se retraen y la superficie de absorción disminuye. Las células intestinales se dividen rápidamente para repoblar el tracto gastrointestinal con células inmaduras resistentes a la infección, pero estas células tardarán un tiempo en reemplazar la función de las infectadas lo cual produce la aparición de diarreas, a veces con resultados fatales.

Por otro lado, HEV es un virus de la familia *Hepeviridae*, sin envoltura, con aproximadamente 20-34 nm de diámetro. La familia incluye dos géneros: *Orthohepevirus*, dividido en 4 grupos (Orthohepevirus A-D) que afectan a diferentes especies de mamíferos y aves y *Piscihepevirus*, que consiste en un solo grupo

que infecta truchas. Los genotipos de HEV que infectan a humanos pertenecen al grupo *Orthohepevirus A*, compuesto por un total de 8 genotipos (HEV1-8) de los cuales 5 (HEV-1, HEV-2, HEV-3, HEV4 y HEV-7) fueron los detectados en humanos. Además, los genotipos HEV-8 y HEV-5 se transmitieron experimentalmente a monos, demostrando la posibilidad de una infección zoonótica.

En cuanto a la distribución global de HEV, las zona geográficas se dividen, en función de la frecuencia de detección, en áreas altamente endémicas, endémicas y no endémicas o esporádicas, haciendo este virus un patógeno ampliamente distribuido. De hecho, es la causa principal de hepatitis entérica en países en vías de desarrollo, especialmente en regiones tropicales y subtropicales, donde las epidemias se han relacionado con el consumo de aguas contaminadas. Además, es particularmente grave en mujeres embarazadas, en las que puede llegar a causar entre un 15-25% de mortalidad cuando la enfermedad es adquirida durante el tercer trimestre de gestación, siendo la diátesis hemorrágica o la hemoglobinuria relacionada con la insuficiencia renal las causas más frecuentes de muerte. Los síntomas descritos de HEV son náuseas, orina oscura, dolor abdominal, vómitos, prurito, dolor en las articulaciones, erupción cutánea y diarrea. Algunos pacientes infectados además presentan fiebre y todos tienen hepatomegalia.

No existe un tratamiento específico para la infección por HEV, sin embargo, el tratamiento antiviral con ribarin o interferón pegilado alfa está indicado para el tratamiento de infecciones crónicas. Existe una vacuna desarrollada en 2011 (Hecolin®) para el genotipo HEV-1, que también demostró ser efectiva contra el genotipo HEV-4. Sin embargo, debido a la falta de datos clínicos solo se aprobó en China, lo cual hace evidente la necesidad de investigaciones que aporten un mayor conocimiento sobre la seguridad y efectividad de la vacunacíon.

Los moluscos bivalvos obtienen su alimento a través del filtrado de pequeñas partículas suspendidas en el agua. Muchas veces estas aguas están contaminadas, haciendo que en el proceso de filtrado los moluscos retengan y concentren patógenos derivados de las aguas residuales. En los últimos años se ha observado que los virus entéricos son los agentes etiológicos más comunes transmitidos por moluscos, siendo la hepatitis y la gastroenteritis las principales enfermedades asociadas a este tipo de contaminación.

Los estudios virales de moluscos basados en técnicas como la RT-qPCR (PCR cuantitativa) reflejan el riesgo que supone para sus consumidores, así como la recirculación de los virus entre la población y las áreas de cultivo. Adicionalmente, aportan información clave para el establecimiento de nuevos valores de corte en futuras legislaciones de carácter microbiológico y

evidencian el impacto de estos patógenos sobre el sector de la acuicultura.

Estudios previos realizados en nuestro laboratorio demostraron el importante papel que juegan algunos virus entéricos tales como NoV o SaV como agentes etiológicos de la gastroenteritis aguda en Glicia. El objetivo principal de la presente tesis doctoral fue la adquisición de mayor conocimiento acerca de AiV y HEV, ambos importantes virus entéricos emergentes. Los estudios realizados durante esta tesis constituyen las primeras investigaciones sobre AiV y HEV realizadas en las principales rías gallegas (las zonas de cultivo más importantes de Europa). Los datos obtenidos se podrán traducir en un aumento de la calidad y seguridad sanitaria del producto además de representar un beneficio indirecto en el sector de la acuicultura, tanto de forma productiva como económica.

Por otro lado, las investigaciones realizadas con muestras clínicas constituyen el primer estudio a prolongado en el tiempo de AiV en muestras de heces en España, estableciendo evidencias sobre la prevalencia del virus en la población gallega.

Teniendo esto en cuenta, el objetivo principal se puede desglosar en cuatro objetivos específicos:

- Evaluar la presencia e incidencia de AIV y HEV en especies de moluscos cultivados y recolectados en Galicia y aptos para el consumo.

- Determinar la epidemiología molecular de AiV y su dinámica poblacional dentro del estudio clínico en el área geográfica de la ciudad de A Coruña.

- Evaluar la diversidad genética de ambos virus y establecer relaciones filogenéticas entre las cepas virales detectadas en moluscos y muestras clínicas.

- Desarrollar un estudio epidemiológico para aclarar el origen de la contaminación presente en las áreas de recolección de mariscos.

En los tres artículos correspondientes al apartado *"3.1 Estudio de los virus entéricos en moluscos gallegos"* (*Article 2. Low prevalence of Aichi virus in molluscan shellfish samples from Galicia (NW Spain); Article 3. Hepatitis E virus genotype 3 in mussels (Mytilus galloprovinciallis), Spain; Article 4. Detection of hepatitis E virus in shellfish harvesting areas from Galicia (NW Spain)*) se determinó la presencia y cuantificación de AiV y HEV en diferentes especies de moluscos bivalvos cultivados en las rías gallegas mediante la técnica de RT-PCR en tiempo real.

Todos los ensayos llevados a cabo en este trabajo han seguido las especificaciones las directrices especificadas por la Organización Internacional para la Estandarización (ISO), con ligeras modificaciones, desarrolladas para el estudio de otros virus entéricos, como son NoV y HAV, en muestras de alimentos y agua.

Esas especificaciones incluyen controles de la eficiencia de extracción y amplificación que permiten evaluar la pérdida de carga viral durante el proceso de extracción, además de estimar la presencia de inhibidores en los procesos de PCR evitando resultados negativos falsos. Todo ello se traduce en una mayor consistencia de los datos obtenidos, aportando seguridad y reproducibilidad para los futuros análisis e interpretaciones.

Las muestras obtenidas de tres rías gallegas diferentes (Ría do Burgo, Ría de Ares-Betanzos y Ría de Vigo), incluían mejillones cultivados y de roca (*Mytilus galloprovincialis*), almejas (*Ruditapes philippinarum y Ruditapes decussatus*) y berberechos (*Cerastoderma edule*), recolectados en diferentes zonas de cultivo.

Se procesaron un total de 249 muestras, de las cuales 168 fueron recogidas a lo largo de 18 meses (desde enero de 2011 hasta junio del 2012) en diferentes puntos de la Ría de Ares-Betanzos y la Ría de Vigo, ambas clasificadas como zonas B (230-4600 MPN *Escherichia coli* por 100g de molusco) de acuerdo con la legislación europea. Los 81 restantes, fueron muestras de mejillón obtenidas de 7 puntos diferentes de la Ría del Burgo, 5 clasificados como B y 2 como C (4600-46000 MPN *Escherichia coli* por 100g de molusco) a lo largo de otros 18 meses (desde octubre de 2010 hasta marzo del 2011).

Los resultados obtenidos en las tres rías mostraron que la prevalencia en Galicia de HEV es más significativa que la de AiV.

Mientras que AiV solo se detectó en un total de 15 (6,0%) muestras, HEV se detectó en 53 (21,2%) de las 249 muestras. Esta predominancia de HEV también se observó en los niveles de cuantificación. Así, los niveles de HEV fueron desde valores no cuantificables (por debajo del limite de detección) hasta 1.1 x 10^5 copias de RNA/gramo de tejido, los de AiV fueron desde no cuantificable hasta 6.0 x 10^3 copias de RNA/ gramo de tejido.

En el norte, HEV se detectó en 18 (20,0%) de las 90 muestras de la Ría de Ares-Betanzos y en 12 (14,8%) de las 81 muestras de la Ría do Burgo, por otro lado, AiV fue detectado en 10 (11,1%) y en 3 (3,7%) muestras respectivamente. En cuanto a la Ría de Vigo HEV se detectó en 23 (29,4%) de las 78 muestras mientras que AiV solo en 2 (2,6%).

Atendiendo a la actividad de las zonas situadas cerca de los puntos de muestro, los resultados de HEV cobran un mayor sentido. Los miles de cerdos criados cerca de las rías producen grandes cantidades de estiércol que pueden estar contaminadas con este patógeno. Esta idea se vio reforzada por los análisis filogenéticos llevados acabo con las muestras positivas para HEV. Estos análisis basados en la región ORF2 mostraron una gran similitud entre las muestras, correspondientes al genotipo 3e, relacionándolas con otras cepas originarias de cerdos y jabalís. Sin embargo, todavía no se han realizado análisis sistemáticos de HEV en el ganado porcino en Galicia para confirmar esta relación, por lo

que se necesitarán más estudios para confirmar esta ruta de contaminación.

Comparando los resultados obtenidos con otras investigaciones previas de nuestro grupo, recogidas en el quinto artículo presentado en el mismo apartado (*Article 5. An overview of 20 years of studies on the prevalence of human enteric viruses in shellfish from Galicia, Spain.*) se pudo observar que la mayoría de las muestras positivas, tanto para HEV como para AiV, estaban contaminadas con otros virus entéricos.

El sexto artículo de esta tesis presentado en el apartado "*3.2 Presencia de Aichi virus en la población gallega"* (*Article 6. Epidemiology of Aichi virus in fecal samples from outpatients with acute gastroenteritis in norwesthern Spain*.) tuvo por objetivo analizar la incidencia de AiV humanos en pacientes afectados por gastroenteritis aguda en la ciudad de A Coruña, y el rol de este patógeno como agente etiológico en el área metropolitana. Los métodos usados en este caso fueron similares a los utilizados para moluscos, detectando y cuantificando el virus con la técnica de RT-PCR en tiempo real, y realizando la secuenciación para los estudios filogenéticos.

Para ello, se analizó la presencia de AiV en pacientes que padecían gastroenteritis aguda y que requirieron ayuda médica en el Complejo Hospitalario Universitario de A Coruña (CHUAC). Los resultados de las muestras clínicas mostraron una prevalencia

de AiV parecida a la detectada en moluscos. De las 2.667 muestras analizadas, AiV se detectó en solo 124 (5%). Sin embargo, la cantidad de monoinfecciones observadas (58,1%) respalda la hipótesis propuesta de AiV como un agente patógeno lo suficientemente virulento como para causar sintomatología que precise atención médica y/o hospitalización.

No se pudo establecer de forma clara una estacionalidad para AiV, detectándose principalmente en los meses fríos y con algunos picos de verano. Factores como las condiciones climatológicas y oceanográficas, los aumentos en los viajes o la estacionalidad del consumo de moluscos podrían influir en estos picos estivales.

Para poder establecer una relación entre cepas virales detectadas a partir de muestras clínicas y de moluscos se llevaron a cabo análisis filogenéticos. Estos análisis basados en la región VP1 de AiV, mostraron que el genotipo A es más común en niños de 0-2, años, mientras que el genotipo B es mas detectado en pacientes de 19-59 años y el único que se detectó en las muestras de moluscos. Estos resultados sugieren que los moluscos podrían estar involucrados en esta deriva del genotipo A al genotipo B con la edad, debido probablemente a los cambios en los hábitos alimentarios producidos a lo largo de la vida.

Sin embargo, es necesario indicar que, aunque los resultados podrían indicar la citada relación entre el consumo de moluscos contaminados y el desarrollo de gastroenteritis, la información

epidemiológica necesaria para establecer de manera fiable esta relación es insuficiente.

Además, las limitaciones de las metodologías de PCR dificultan aún más esta tarea, ya que esta técnica solo tiene en cuenta la amplificación de los ácidos nucleicos y no detecta por tanto las partículas infectivas. Es por ello que el riesgo real de infección y el papel de los moluscos bivalvos en la transmisión de ambos patógenos estudiados sigue siendo desconocido, y se necesitarán más investigaciones para clarificar este papel.

En resumen, los resultados expuestos revelan la aparición de patógenos emergentes en las zonas de cultivo de Galicia, una de las áreas de producción de moluscos bivalvos más importantes del mundo. Por otro lado, la similitud genética de las cepas de AiV y HEV detectada entre las muestras clínicas y los moluscos sugiere que este tipo de alimento puede constituir una ruta de transmisión de este patógeno. En este sentido, creemos que los hallazgos obtenidos durante este trabajo pueden contribuir a llenar un cierto vacío de conocimiento existente en este campo de estudio.

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